The development of a nano-sized eggshell and titanium dioxide desensitising paste to re-mineralise damaged teeth.

Submitted in fulfilment of requirements of the degree of
Doctor of Dental Technology: Dental Technology in the Faculty of Health Sciences at the Durban University of Technology.

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May 2019

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The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Abstract

Dentin hypersensitivity [DH] is a common occurrence and notable painful condition among dental patients. Clinically, the pain associated with DH is caused by external stimuli such as thermal, tactile, osmotic or chemical changes from open dentinal tubules. Traditionally, the treatment of DH is the use of at-home desensitising toothpaste. While there is a variety of desensitising paste such as Sensodyne® and Colgate sensitive Pro-Relief ™ to treat DH, the dentin tubule remineralising characteristics of these paste are, however, limited in an acidic environment which could result in DH relapse. The limiting abilities of these desensitising paste prompt this study to develop a desensitising agent using nano-sized eggshell-titanium dioxide (EB@TiO_2) as an active ingredient in the management of DH.

A quantitative research design and an experimental research strategy were adopted. The research design included three phases. In phase one of this study, different characterisation techniques such as Fourier Transform Infrared Spectroscopy (FTIR); X-ray Diffraction (XRD); Energy Dispersive X-ray Spectroscopy (EDX) and Scanning Electron Microscope (SEM); High Transmission Electron Microscopy (HRTEM); and Thermo-Gravimetric were used to confirm the modification of EB@TiO_2. Phase two, on the other hand, assesses the suitability of the EB@TiO_2 as an oral healthcare product by examining its cytotoxicity and antibacterial properties. By contrast, phase three investigated the quality of the EB@TiO_2 as a new approach to the management of DH. Particularly, the acid resistant, abrasivity, and remineralisation characteristics of EB@TiO_2 were studied using bovine and eggshell model, respectively. Different analytical technique such as pressure sensor, gas displacement test, Atomic Force Microscope, Raman Spectroscopy, SEM, and EDX were used to examine the product quality of EB@TiO_2 in comparison with some commercially available paste. In addition, a mathematical model was used to predict the duration and rate of remineralisation of EB@TiO_2. Both descriptive and inferential statistics were used to present the data (P=0.05). The validity of the study was achieved following SANS 1302 (2008) requirement for preparation, developing, and testing toothpaste. The reliability was determined via reproducibility and repeatability of tests.

Paper I and II examines the effectiveness of commercially available toothpaste in the prevention of tooth decay, using eggshell powder as a substitute for the human tooth. Paper I established that eggshell model can be used as a substitute for the human tooth to study the acid resistant properties of toothpaste. The salient point of the paper is that all the tested toothpaste were effective against erosive attacks. However, the eggshell alone without the protective covering of toothpaste showed limited acidic resistance.

Paper II established that acid resistance properties of EB@TiO_2 were significantly better than eggshell alone. The paper confirmed that modifying eggshell with titanium dioxide improved its acidic resistance characteristics.

Paper III and IV evaluate the acidic resistance of EB@TiO_2 using a bovine model. Paper III and IV established that the protective effects of EB@TiO_2 were superior to the tested commercially available toothpaste. The FESEM, AFM, and Raman test further confirmed that EB@TiO_2 offer better protection on the tooth enamel.

Paper V and VI assess the occluding capabilities of EB@TiO_2 and eggshell alone in comparison with other desensitising toothpaste. Paper V confirmed that there was complete remineralisation of the dentin tubules in the samples treated with EB@TiO_2. At higher magnification, the particles of EB@TiO_2 were very much evident. The EDX spectrum reveals that the Ti peaks observe before and after post acidic treatment were comparable.

Paper VI established that occlusion of EB@TiO_2 was highly effective in an acidic environment, as occluded tubules remained intact post-acidic treatment. In addition, the cytotoxicity study identified that EB@TiO_2 had little effect on the NIH 3T3 cell line even at the highest concentration of 100µg/ml.

Manuscript I assess the occluding capabilities of EB@TiO_2 in comparison with a known occluding desensitising agent (Pro-Argin and NovaMin). Manuscript I established that as the brushing days increase the remineralisation or dentin tubule occluded by each respective desensitising agent improved. It was found that the occluding capabilities of EB@TiO_2 were more superior to both Pro-argin and NovaMin products in both saliva and without saliva.

Manuscript II described the use of the logistic equation to predict the remineralisation of the EB@TiO_2. Manuscript II established that the logistic equation effectively predicted the remineralisation trends of EB@TiO_2 and Pro-argin toothpaste (Colgate Pro-relief).

Manuscript III assesses the abrasivity of EB@TiO_2 in comparison with calcium carbonate, and hydrated silica containing toothpaste. Bovine enamel specimen was used for the in vitro experiment. Manuscript III established that enamel loss from the brushed surface, regardless of the sample group, were statistically different when compared to the covered surface. The study found that the abrasivity of EB@TiO_2 were comparable with the calcium carbonate toothpaste. It was also established that EB@TiO_2 was less abrasive when compared against hydrated silica containing toothpaste.

In conclusion, the experimental finding has exhaustively provided evidence on the suitability of EB@TiO_2 as an active ingredient in toothpaste formulation. The study, therefore, provides new evidence and approach for the management of DH, particularly in low-income countries where the cost of oral healthcare may be too high.

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Declaration

I, Stanley Chibuzor Onwubu, hereby declare that this thesis is wholly my own work, and that all the references to the best of my knowledge, are accurately reported. This work has not been submitted for a degree at any other university, and that its only prior publication was in the form of conference papers, journal publications, and book chapters' as listed below.

CONFERENCE PAPER AND ABSTRACTS ARISING FROM THIS STUDY

Onwubu, S.C., Mdluli, P.S., Singh, S. and Bharuth, V. 2017. An in vitro examination on the effectiveness of commercial toothpaste in the prevention of tooth decay using eggshell as the substitute for human tooth. Abstract accepted 318th International Conference on Medical and Health Sciences (ICMHS) Cape Town, South Africa 28th-29thDecember2017.

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JOURNAL PAPERS PUBLISHED AND SUBMITTED ARISING FROM THIS STUDY

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Signature Date

Dedication

Dedicated to my loving parents, Mr and Mrs Sylvester Onwubu. Your love and support inspired in me the pursuit of excellence and the desire to learn more.

Acknowledgements

The completion of this thesis was made possible through the support, encouragement and assistance from my family, friends and colleagues. Indeed, the recognition that this work may receive is not possible through sheer effort, but to all those who made it possible. I am particularly indebted to the following individuals:

Prof Mdluli Phumlane – for spending many hours supervising this thesis. It was through his patience, consistent encouragement, enthusiasm, constructive criticism, freedom, and insightful guidance throughout my study that I was able to complete this thesis timeously. Your technical expertise and financial assistance provided a solid foundation in realising the end product of this thesis

Prof Shenuka Singh – for your thoroughness in correcting and structuring this dissertation. Your deep knowledge in research methodology together with your expertise in public health dentistry is evident throughout this thesis. I wholeheartedly thank you.

My Siblings – your prayers were never in vain. Your consistent calls and encouragement were the ointment that grease my hands throughout this thesis.

Mrs. Thakur and Dr. Thakur family – the flame of your friendship, continual support and encouragement ignited me to persevere in writing this thesis. Maleni, you are the best friend anyone would wish for. I am so grateful and forever indebted to your benevolence and kindness.

Miss Makgobole Mokgadi – you became the pillar of friendship in my life. Your support and will power echoed throughout the writing of this thesis.

Prof Ashley Ross – the most supportive chair of faculty research committee with much foresight. Thank you for opening the doors to your office. Your support and kindness is most appreciated.

Mr. Philani Ngcobo – never known any better man than you. Your support and friendship are immeasurable. You open the doors to your laboratory and afford me the opportunity to prepare my samples at no cost. The smile and radiance that came out of your face is very much evident in this thesis.

Mr. Sugen Reddy – my run to person. I am grateful for your assistance and for your words of encouragement during the most difficult time in this study.

Finally, no acknowledgement would be complete without a wholehearted thanks to: Mr Jimmy Chetty (Department of Chemistry, DUT), Dr. Sanele Nyembe (MINTEK), Dr. Henrietta Van De Wat (MINTEK), Mr. Kabange Kasumbwe Laurel (Department of Biotechnology, DUT), Dr. Obiora Collin (Research office, DUT), Mr. Vishal Bharuth (Microscopy and Microanalysis Unit, UKZN), for their expertise in completing this dissertation.

Acronyms

ANSI/ADA	American National Standards/American Dental Association
AFM	Atomic Force Microscope
ASTM	American Society for Testing and Materials
DH	Dentin Hypersensitivity
DUT	Durban University of Technology
EB@TiO_2	Eggshell-titanium dioxide
EDX	Energy Dispersive X-ray Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
HRTEM	High Resolution Transmission Electron Microscope
IUPAC	International Union of Pure and applied Chemistry
ISO	International Standard Organisation
RPM	Revolution per Minute
SEM	Scanning Electron Microscopy
SPSS	Statistical Package for Social Science
TGA	Thermo-Gravimetric Analysis
UKZN	University of KwaZulu-Natal
OTC	Over the counter
XRD	X-ray Diffraction

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Chapter One - Introduction

1.1 Background and context of the Study

Dentin Hypersensitivity [DH] is a common occurrence and under extreme conditions when dentine is exposed to the oral cavity patients will experience a short, sharp pain (Addy 2000; Canadian Advisory Board on Dentin Hypersensitivity 2003; Panagakos, Schiff and Guignon 2009). Clinically, patients become more susceptible to external stimuli such as thermal, tactile, osmotic or chemical changes due to open dentinal tubules (Canadian Advisory Board on Dentin Hypersensitivity 2003). Traditionally, and as reported by Schiff *et al.* (2009), Low, Allen and Kontogiorgos (2015), and Arnold *et al.* (2016), the first-line of 'at-home' treatment is to use desensitising toothpaste. Professional 'in-office' products and procedures such as the use of varnishes, precipitants and the placement of restorative materials are often considered clinically for the control of DH (John *et al.* 2015). Although at-home desensitising toothpaste and professional in-office treatment have been clinically proven to provide some relief to patients, Markowitz and Pashley (2008) and Pashley *et al.* (2008), have documented that these treatments are ineffective in the overall treatment of DH.

According to Anusavice and Antonson (2013: 251), the abrasiveness of a desensitising dental toothpaste may adversely impact on the exposed cementum and dentine surfaces. Hence, the ideal toothpaste should provide the greatest possible cleaning action on tooth surfaces with the lowest possible abrasion rate (Anusavice and Antonson 2013). Several studies (Garcia-Godoy, Garcia-Godoy and Garcia-Godoy 2009; Schiff *et al.* 2009; Yang *et al.* 2016; Hirsiger *et al.* 2019) on desensitising dental toothpaste, however, suggests that using arginine together with calcium carbonate could be used in treating DH. Using calcium carbonate to treat DH, therefore, prompts this study to develop a desensitising paste using eggshell and titanium dioxide in the remineralisation of enamel lesions for the treatment of DH.

Moreover, it is well documented in the literature (Gergely *et al.* 2010; Gao and Xu 2012; Mosaddegh and Hassankhani 2013; Feng *et al.* 2014; Baláž *et al.* 2015; Ibrahim *et al.* 2015; Onwubu *et al.* 2017a) that calcium carbonate, which is generally mined from rocks (Boron 2004; Oliveira, Benelli and Amante 2013), is present naturally in eggshells (95% by weight). A recent study demonstrated that a dental abrasive material made from eggshells was effective in the polishing of dental acrylics (Onwubu *et al.* 2017b). Similarly, research work conducted by Nakashima *et al.* (2009), and Rahardjo *et al.* (2015) showed that the inclusion of nano-sized calcium carbonate in dentifrice re-mineralises enamel lesions. It can, therefore, be gathered that the physical characteristics of eggshells, namely crushable, porous, and lightweight, together with its abundant availability and high purity content supports its use in developing a desensitising dental toothpaste to treat DH.

Equally significant, Cutler (2014) suggested that nano-sized titanium dioxide and dental abrasive agents can be used together in occluding open dentinal tubules to effectively reduce DH. Despite the unprecedented advances made in nanotechnology in diagnosing and preventing disease in health care, drug delivery, bone augmentation and replacement (Perán *et al.* 2013; Solano-Umaña, Vega-Baudrit and González-Paz 2015) there is limited evidence on using nanomaterials to treat DH. This study, therefore, aims to use nano-sized eggshells and titanium dioxide to develop a desensitising dental toothpaste to treat DH.

1.2 Research Problem

Dentin Hypersensitivity [DH] if left untreated will negatively affect the quality of life for dental patients'. In particular, and as Schiff *et al.* (2009) pointed out, DH sufferers tend to modify their habits by eliminating certain foods and drinks from their regular diets and they become non-complaint with specific at-home care recommendations such as tooth brushing. The authors further noted that there is a higher accumulation of dental plaque in patients that are non-compliant with specific at-home care recommendations from their oral care providers (Schiff *et al.* 2009). Consequently, caries formation, gingival inflammation, and periodontal problems are likely to increase. Several authors (Orchardson and Gillam 2006; Cummins 2009; Panagakos, Schiff and Guignon 2009; Yang *et al.* 2016) have therefore advised using a desensitising paste to physically occlude exposed dentine tubules.

Over the last decades, the oral care industry has witnessed the proliferation of different occlusion materials for treating DH. Among these materials, the use of potassium oxalates (Cunha-Cruz *et al.* 2011), sodium fluoride (Pandit, Gupta and Bansal 2012), strontium salt (Saeki *et al.* 2016), amorphous calcium phosphate containing casein phosphopeptide (Babu, Subramaniam and Teleti 2018) calcium glycerophosphate (Zalite and Locs 2017), and calcium carbonates (mainly as abrasive agents) have gained significant interest as an occlusion materials. Although the aforementioned occlusion materials have been reported to provide some relief to patients, the dentine tubules occluded by some of these materials are reported to be superficial with limited infiltration depth-which could be readily re-exposed in an acidic environment (Arnold, Prange and Naumova 2015). Thus, short-living the treatment effects and subsequently resulting in DH relapse (Yu *et al.* 2016). Given the limited drawbacks, developing desirable biomaterials for DH becomes highly critical to not only efficiently occlude the exposed dentine tubules, but also remain effective in an acidic environment (Yu *et al.* 2016).

Although other techniques such as lasers and iontophoresis have been proposed in the literature to manage DH (Cartwright 2014), they are, however, expensive, and complex to use- making it unfeasible in long term management of DH (Sgolastra *et al.* 2011; Mao and Toby 2013; Cartwright 2014). Consequently, Schmidlin and Sahrmann (2013), suggested a non-invasive, non-hazardous, reversible, easy to perform, and inexpensive material for the management of DH. However, there is no much research involving the use of novel materials such as eggshell waste that could be cost-effective. This is particularly relevant in countries with low socioeconomic status, and suffering from neglected oral health care (Ramphoma 2016).

Furthermore, there is no published literature that has examined the use of a modified nano-sized eggshells as active ingredients for managing DH. Moreover, scholars (Kovtun *et al.* 2012; Wang *et al.* 2014) have suggested that the combination of nanomaterials offers better treatment strategy for the management of DH. It is imperative to assume that the proposed use of waste eggshells material would not only provide an affordable and accessible strategy to manage DH, but could also help address the challenge and cost of accessing oral health in most low-income countries.

1.3 Aim

The study aimed to develop and evaluate *in vitro* the quality of a modified nano-sized eggshell and titanium dioxide (EB@TiO_2) composite as the active ingredients in the management of DH.

1.4 Objectives of the Study

The objectives of this study will be done in three phases, namely:

(1) To develop and characterise modified Nano-sized eggshell-titanium dioxide desensitising agents;

(2) To determine biocompatibility and conduct microbial assessment of titanium oxide and eggshell base desensitising agents; and

(3) To establish the product-based quality of titanium oxide and eggshell base in terms of ‘fitness for purpose’.

It should be noted that phases one and two will test the properties of the titanium oxide and eggshell material in line with International Standard Organisation (South African National Standards 1302: 2008) requirement for desensitising toothpaste.

Objective One: To develop and characterise modified Nano-sized Eggshell-Titanium oxide (EB@TiO_2) desensitising agents.

The sub-objectives are:

1.4.1 To determine the physical characteristics of EB@TiO_2 desensitising agents in terms of crystallisation; particle size; and phase, and shape in order to establish the bond strength characteristics of the newly developed EB@TiO_2 desensitising agents.

1.4.2 To conduct thermal analyses of EB@TiO_2 desensitising agents using thermo-gravimetric analysis (TGA) in order to determine the thermal stability and degradation of the product.

1.4.3 To determine the chemical constituents of EB@TiO_2 desensitising agents using Energy Dispersive spectroscopy (EDX) to ensure that product is free of hazardous substances.

1.4.4 To use a viscometer in order to analyse the viscosity and rheological properties of EB@TiO_2 desensitising agents to ensure consistency in the flow properties of the product.

Objective Two: To determine biocompatibility and conduct microbial assessment of Titanium oxide and Eggshell base (EB@TiO$_2$) desensitising agents.

The sub-objectives are:

1.4.5 Using a Bacteria strain *E.coli* (ATC 25922 strain) and *B. Cereus* (ATC 10876) to assess the antibacterial properties of EB@TiO$_2$ desensitising agent in line with ISO 21149 and ISO 16212 for cosmetic products.

1.4.6 Using NIH 313 and BHK21 cell lines, the toxicological profile of EB@TiO$_2$ desensitising toothpaste will be characterise to ascertain its cytotoxicity in line with ADA 1979 standards.

Objective Three: To establish the product-based quality of Titanium oxide and Eggshell base (EB@TiO$_2$) in terms of 'fitness for purpose'

The sub-objectives are:

1.4.7 To determine the abrasivity of EB@TiO$_2$ desensitising toothpaste on bovine enamel in line with recommended standard by American Dental Association (ADA) and the International Standard Organisation (ISO).

1.4.8 To evaluate the acid resistant properties of EB@TiO$_2$ using a scanning electron microscope (SEM), and atomic force microscope (AFM), Raman spectroscopy, and pressure sensor in order to determine its suitability for enamel remineralisation.

1.4.9 To examine the effectiveness of EB@TiO$_2$ desensitising toothpaste in occluding open dentine tubules using a scanning electron microscope (SEM) and ImageJ software.

1.4.10 To evaluate the occluding characteristic of EB@TiO$_2$ in reducing DH compared with commercial toothpaste containing Pro-Argin and NovaMin with or without saliva

1.4.11 To use a mathematical equation to forecast the duration of dentin tubule remineralisation on bovine teeth treated with EB@TiO$_2$ desensitising toothpaste.

1.5 Research Hypotheses

1.5.1 H_0: There are is no significant difference in the abrasion rate of EB@TiO_2 and commercially available toothpastes on tooth enamel.

1.5.1 H_1: There are significant differences in the abrasion rate of EB@TiO_2 and commercially available toothpastes on tooth enamel.

1.5.2 H_0: There will be no difference in the dentine tubules occlusion pre- and post-acidic exposure of the specimens treated with EB@TiO_2.

H_1: There will be significant difference in the dentin tubules occlusion pre- and post-acidic exposure of the specimens treated with EB@TiO_2 desensitising toothpaste.

1.5.3 H_0: There is no significant difference in the protective effect of EB@TiO_2 against erosive acids when compared against commercially available toothpastes.

H_1: EB@TiO_2 will offer more protection against erosive acids compared to commercially available toothpastes.

1.6 Rationale/Significance of the study

Calcium carbonate products to treat DH has generated some research interest (Kleinberg 2002; Nakashima *et al.* 2009; Cutler 2014; Rahardjo *et al.* 2016; Budde, Gerard and Gane 2017). For example, Kleinberg (2002) suggested that arginine, bicarbonate, and calcium carbonate can physically plug and seal exposed dentin tubules thereby effectively relieving DH. Moreover, Nakashima *et al.* (2009), and Rahardjo *et al.* (2015) indicated that nano-sized calcium carbonate, when used in toothpastes are highly effective in remineralising enamel lesions. Similarly, Cutler (2014) suggested that nano-sized titanium dioxide and dental abrasive agents can be used together in blocking exposed dentinal tubules to effectively reduce DH. From an environmental sustainability and management perspective, and as argued by Abdulrahman *et al.* (2014), using eggshell waste material to manage DH will strengthen the economic benefits associated with using natural waste material, which is high on the global agenda for a greener environment. This argument is supported by Yazıcıoğlu and Ulukapı (2014) who pointed out that a low cost, affordable, feasible, and sustainable products need to be developed to repair and maintain oral health.

It is highly anticipated that should EB@TiO_2 be successfully developed, the product will contribute to cost-effective alternatives for the population at large. This reechoes the assertion made by Cartwright (2014) that future strategy to manage DH should be cost-effective. Essentially, this study, therefore, aligns with the national imperatives to identify cost-effective alternatives to self-care management of DH (National Planning Commission 2012). More importantly, this study will provide important baseline data for further research in this area. Although in early stages, this study can ultimately contribute in the long term to oral health planning on the management of DH, this will also help address the call made by Cartwright (2014) that future management of DH should be more permanent. In addition, this study can also contribute to dental materials research in the country and in other regions of the world. This is also in accordance with the paradigm shift in dentistry from a treatment-oriented approach to a more preventative oral healthcare (Glick *et al.* 2016).

1.7 Structure of the Thesis

The thesis will be divided into six chapters. Chapter one provides extensive background and context of the study. This steered the chapter towards the aim and objectives, the research rationale/significance, and hypotheses of the study.

Chapter 2 presents an overview of the literature on the structure of the human tooth. This will include a discussion on the aetiology, histopathology, and trends in the management of DH. Subsequently, the introduction and review of eggshell and titanium dioxide composition, characteristics and their various applications follow.

Chapter 3 presents the theoretical framework for the study. In particular, the social justice principle was elaborated upon as the underpinning motivation for the use of eggshells. This will include an explanation of the hydrodynamic theory proposed by Brännström as the guiding theory to validate EB@TiO_2.

Chapter 4 describes the research design and methodology by detailing the quantitative research paradigm and experimental research design that is to be adopted in this study. This will include an explanation of the three research phases adopted.

Chapter 5 provides a critical overview of the study. In particular, the interrelationship in the published and unpublished manuscript was presented with a view to addressing the research hypotheses.

Chapter 6 forms the final chapter and will provide the conclusions drawn from the study. It will also identify any limitations and consider future directions for this research.

Chapter Two - Literature Review

The chapter reviews literature related to the treatment and management of dentine hypersensitivity [DH]. The review presents an overview of the structure of dentine and enamel, particularly that of the human and bovine tooth. This will include a discussion on the aetiology, histopathology, and trends in the management of DH. Subsequently, the introduction and review of eggshell and titanium dioxide composition, characteristics and their various applications follow. Overall this literature review is structured into eight sections. Section one discusses the structure of the human tooth in terms of its composition, and properties. The use of bovine tooth as an alternative for the human tooth in an *in vitro* dental studies will be explained. Section two describes the concepts of tooth loss, factors that influence tooth loss, mechanism, and management of DH.

Section three describes the use of nanomaterials in the management of DH with a particular emphasis on mesoporous and nanohydroxyapatite materials. Section four introduces eggshell powder and titanium dioxide as the new approach for DH management. Here their characteristics and applications will be explained. This section will also elaborate on the toxicological of concerns nanomaterials. Section five takes a departure from the DH management by exploring the techniques of preparing EB@TiO_2. Here, the science of mechanochmistry will be introduced and elaborated upon.

Section six details the requirement for densensitising toothpaste. This will include a discussion on the abrasivity and erosive resistant requirement of toothpaste. It is anticipated that this will help to support the development of EB@TiO_2-EB desensitising toothpaste. A discussion follows on the different techniques for validating dentine remineralisation, enamel demineralisation, and abrasivity. The chapter concludes with an explanation of the proposed logistic equation, particularly as it is a crucial factor for developing a predicting model for evaluating the remineralisation potential of EB@TiO_2 desensitising toothpaste.

2.1 Overview and structure of the human tooth

Several studies (Yahyazadehfar, Bajaj and Arola 2013; Sui *et al.* 2014; Zhang *et al.* 2014; Healy 2016) have reported that the human tooth is composed of dentin, enamel, cementum, and pulp. According to Zhang *et al.* (2014), the first three (enamel, dentin, and cementum) constitute the hard tissue of the human tooth and are characterised by their unique mechanical properties, structure and chemical composition. With reference to the dentin, (Zavgorodniy, Rohanizadeh and Swain 2008) pointed out that the human dentin is a hard, elastic, avascular connective tissue which forms the bulk of the tooth. Structurally, and as revealed by Nanci (2003), Lopes *et al.* (2009), and Healy (2016) dentin is located between the exterior enamel and the interior pulp, and functions to support the very highly mineralised enamel in withstanding the forces of mastication without fracture.

From an anatomical context, dentin consists of microscopic dentinal tubules that are about 2-4 µm in diameter (Figure 2-1). Microscopically, Ross and Pawlina (2006), Bath-Balogh and Fehrenbach (2011), and Fehrenbach and Popowics (2015) revealed dentinal tubules radiate outward through the dentin from the pulp to the exterior cementum. As a consequence, Bath-Balogh and Fehrenbach (2011) asserted that dentin has a degree of permeability, which can increase its sensation of pain.

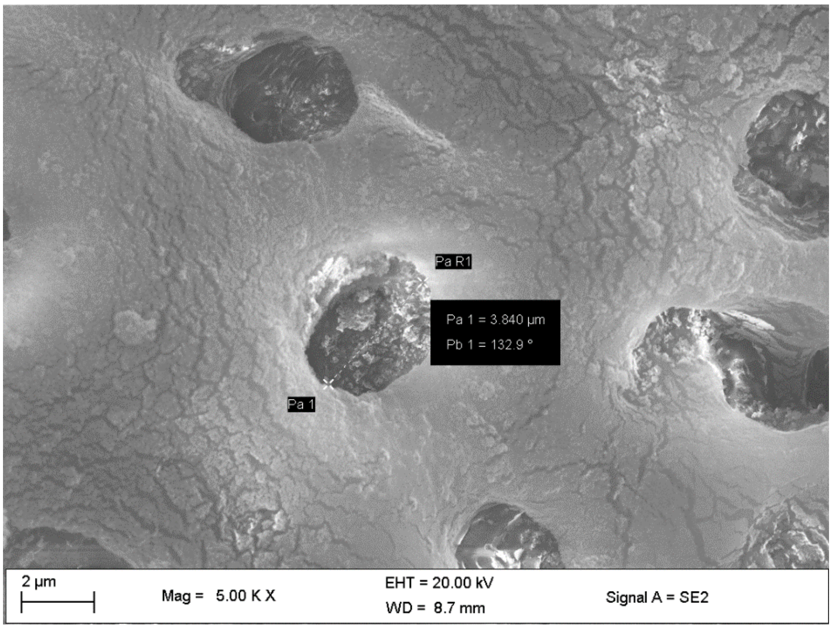

Figure 2- 1: Diameter of open dentin tubules

In terms of dentin composition, studies (Zavgorodniy, Rohanizadeh and Swain 2008; Lopes *et al.* 2009; Healy 2016) have documented that dentin is a hydrated nano-composite that comprises of hydroxyapatite $[(Ca_2(PO_4)_6 (OH)_2)$ mineral crystallite with a thickness of (~ 5nm) and (~ 45% by volume) distributed in a scaffold of type 1 collagen fibrils (~ 50-100nm diameter) and about 20% fluid. In contrast to the dentin, Ramakrishnaiah *et al.* (2015), Zhang *et al.* (2014), and Healy (2016) outlined that the enamel is dense, highly mineralised, hard, and brittle. More so, studies (Nanci 2003; Bath-Balogh and Fehrenbach 2011; John *et al.* 2015) have stressed that the enamel is susceptible to demineralisation due to its high mineral constituent of 96% by weight of the inorganic structure (Table 2-1). This is concerning as the enamel which is the outmost layer of the crown of a tooth protects the underlying dentin and pulp tissue (Desoutter *et al.* 2014).

Table 2- 1: Comparison of the structure and composition of human enamel and dentin

Dental tissue	Enamel	Dentine
Composition	96% inorganics, the rest are water and organics	65%-70% minerals, the rest are organics
Microstructure	Enamel rods, enamel rod sheath	Dentinal tubule, peritubular dentin, intertubular dentin

More recently, Ekambaram and Yiu (2016) noted that the enamel does not have the capacity to regenerate and or repair itself. Equally concerning, other studies (Addy and Smith 2010; Miglani, Aggarwal and Ahuja 2010; Borges, Barcellos and Gomes 2012; Taha *et al.* 2015) have reported that the demineralisation or loss of the enamel exposes the underlying dentin to the external environment, which in turn leads DH. Hence, a thorough knowledge regarding DH aetiology and predisposing factors is inevitable for its effective treatment and management.

According to Yassen, Platt and Hara (2011: 273), specimens generated from human teeth are generally preferred for *in vitro* and *in situ* dental research, since they allow for testing of the study hypothesis in a more clinically relevant substrate. The early work of Mellberg (1992), however, noted that some disadvantages and limitations exist with the use of human teeth. For example, Mellberg (1992) alleged that the human teeth are often difficult to obtain in sufficient quantity and with adequate quality because many are extracted due to extensive caries lesions and other defects. Similarly, Zero (1995) argued that it can be challenging to control the source and age of the collected human teeth, which may lead to larger variations in the outcome measures of the study. He further elaborated that the relatively small and curved surface area of human teeth could be a limitation for specific tests requiring flat surfaces of uniform thickness (Zero 1995). In addition, Rueggeberg (1991) and Skene (2002) highlighted a concern in their asserted view that the increase in awareness of infectious disease, and ethical issues has restricted the use of human teeth. As a consequence, Yassen, Platt and Hara (2011) noted that alternative substrate has been proposed and used in dental research.

2.1.1 Bovine tooth as a substitute for human tooth

As revealed in the literature (Abuabara *et al.* 2004; Fonseca *et al.* 2004; Tanaka *et al.* 2008; Lopes *et al.* 2009; Yassen, Platt and Hara 2011; Field 2012), several types of non-human teeth particularly that of bovine and ovine have been utilised as substrates for *in vitro* experiments. From a dental perspective, Tanaka *et al.* (2008), Yassen, Platt and Hara (2011), and Field (2012) contended that dental studies commonly use bovine teeth as substitutes for human teeth. In reviewing the literature on the differences and similarities between human teeth and bovine, and as illustrated in Table 3-2, it is worth noting that there are no significant differences found between bovine and human teeth overall. This resonates with the work recently conducted by Costa *et al.* (2015) that the radicular dentin morphology of human and bovine primary teeth and root are similar in terms of the diameter of the dentine tubules.

Moreover, unlike human teeth, Yassen, Platt and Hara (2011: 273) hinted that bovine teeth with a more uniform composition are easy to obtain in large quantities. They also noted that bovine teeth have a relatively large flat surface, which is free of caries lesions and other defects. The aforementioned studies, therefore, suggest that bovine enamel could be used *in vitro* in assessing the remineralisation of enamel and dentin.

Additionally, Fonseca *et al.* (2004), and Tanaka *et al.* (2008) acknowledged that that ethics committees are encouraging the use of bovine as an alternative for human teeth. Consequently, this has promoted its use by most researchers, since they are easily obtained. A noteworthy point is due to the ethical issues of obtaining human teeth, bovine enamel and dentin will be used in this study. As such, the efficacy of the newly developed EB@TiO_2 desensitising toothpaste in remineralising damaged teeth will be evaluated *in vitro* using bovine enamel and dentin models. The next section, therefore, reviews the factors that influence tooth loss, particularly the demineralisation of enamel.

Table 2- 2: Comparison of the structure and composition of human and bovine teeth (adapted by the researcher)

Parameters	Similarities	Differences
Morphology	1. No significant differences in the mean dentinal tubules between bovine and human coronal dentin (Schilke *et al.* 2000). 2. No significant difference in the number of open dentin tubules between human and bovine sclerotin dentin (Camargo, Marques and de Cara 2008).	1. The diameter of bovine crystallities is larger than that of human teeth in a ratio 1.6:1 (Arends and Jongebloed 1978). 2. The number of tubules per square millimetre, regardless of the region, is significantly higher in human when compared against bovine dentin (Lopes *et al.* 2009).
Chemical composition	1. Presence of inorganic pyrophosphate in both bovine and human teeth(Fleisch *et al.* 1968). 2. The Ca/P ratio in demineralised enamel surface, as well as the remineralisation characteristics are the same in both bovine and human teeth (Feagin, Koulourides and Pigman 1969). 3. No significant difference in fluoride uptake between etched human and bovine enamel (Gwinnett, Buonocore and Sheykholeslam 1972). 4. No significant difference in carbonate content between human and bovine enamel at different stages of their development (Sydney-Zax, Mayer and Deutsch 1991). 5. No significant difference in the pH between bovine and human teeth (Camargo *et al.* 2006). 6. Overall, enamel matrix proteins isolated from human teeth showed similar amino composition to that of bovine teeth (Fincham 1980).	1. Calcium content by weight of bovine and human enamel are 37.9% and 36.5%, respectively (Davidson, Boom and Arends 1973). 2. Bovine enamel has more homogenous calcium distribution when compared against human teeth (Davidson, Boom and Arends 1973). 3. Differences in fluoride uptake by sound permanent human teeth and primary bovine enamel (Loertscher 1973). 4. Bovine teeth have significantly higher calcium ions release compared to human teeth (Camargo *et al.* 2006).
Physical properties	1. No significant difference in the polishing degree as well as refractive indices between bovine and human teeth (Spitzer and Ten Bosch 1975; Putt, Kleber and Muhler 1980). 2. No substantial differences between the luminescence of human and bovine teeth (Spitzer and Ten Bosch 1976).	1. The absorption peak of bovine enamel at 270nm is however, three times higher than human enamel (Spitzer and Ten Bosch 1975). 2. The mean translucency values of bovine enamel, bovine dentin, human enamel, and human dentin are 14.7, 15.2. 18.7, and 16.4, respectively (Yu, Ahn and Lee 2009).

	3. No significant difference in the mineralised and demineralised human and bovine teeth with regards to their ultimate tensile strength or modulus of elasticity (Sano *et al.* 1994). 4. No significant difference in the hydraulic conductance as well as diffusional water influx between human and bovine teeth (Schmalz *et al.* 2001). 5. No significant difference in the radiographic density and hardness between human and bovine teeth (Tanaka *et al.* 2008).	

2.2 Aetiology of DH

As documented in the literature (Addy 2005; Orchardson and Gillam 2006; Borges, Barcellos and Gomes 2012), DH develops in two phases, namely: lesion localised and lesion initiation. Miglani, Aggarwal and Ahuja (2010), Davari, Ataei and Assarzadeh (2013), and John *et al.* (2015) mooted that lesion localisation occurs by loss of protective covering over the dentin. They, together with other authors (Rahardjo *et al.* 2016; Saeki *et al.* 2016; Salahi, Ghanbari and Moosaali 2016), have elaborated that the dentin covering enamel can be lost through attrition, abrasion (Figure 2-2A), erosion (Figure 2-2B) and abfraction. Other causes for enamel loss includes gingival recession, which can be due to toothbrush abrasion, pocket reduction, tooth preparation for a crown, excessive flossing or secondary periodontal diseases (Saeki *et al.* 2016; Salahi, Ghanbari and Moosaali 2016). The aforementioned authors, however, acknowledged that for DH to occur, the lesion localisation has to be initiated.

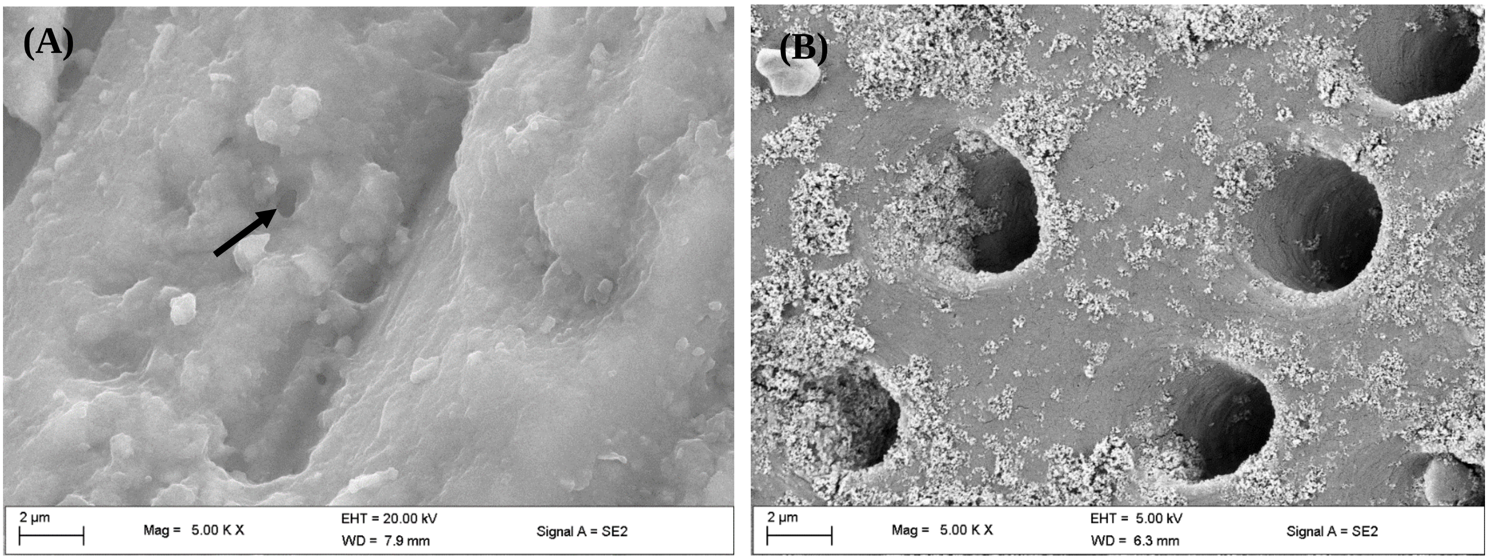

Figure 2- 2: Representative SEM micrograph for (A) Dentine surface abraded with silicon carbide paper for 60s; (B) pre-treatment of dentine surface exposed 1wt. % citric acid solution for 30min. (Original magnification x 5000). Arrows pointing to exposed tubules (Image developed by the researcher).

John *et al.* (2015: 27) have outlined a number of predisposing factors that initiate DH. These include gingival recession, tooth wear, lifestyle, behaviours, oral self-care habits, and the pH of the oral environment; which could be related to dietary as well as xerostomic conditions (John *et al.* 2015). They also noted that erosive agents are important agents in the initiation and progression of DH. As Frederick, DeLaurenti and Olson (2009) explain, erosive agents tend to remove the enamel or open up the dentinal tubules. The said authors pointed out that these erosive agents can be either exogenous dietary acids or endogenous acids. Their work supports the early report of Stoodley *et al.* (2007) who clarified that exogenous dietary includes carbonated drinks, citrus fruits, wines, yogurt, and professional hazards (workers in battery manufacturing, and wine testers), whereas endogenous acid results from gastroesophageal reflux or regurgitation.

Concerning however, the early report by Ling and Gillam (1996) noted that endogenous conditions are characterised by generalised erosion of the palatal surfaces of the maxillary anterior teeth, which could initiate DH. Similarly, Field (2012) moots that erosive agents promote the cycle of demineralisation and remineralisation of the tooth structure (Figure 2-3). His work supports the early report of Moss (1998) who noted that the alteration in the equilibrium of demineralisation and remineralisation negatively affect the structure of the tooth. He emphasised that excess demineralisation result in the loss of the tooth structure, which may trigger DH. The effect of erosive agents on loss of tooth structure is pertinent to this study, particularly as it underpins the motivation for a new biomaterial with high acidic resistant characteristic in an oral environment.

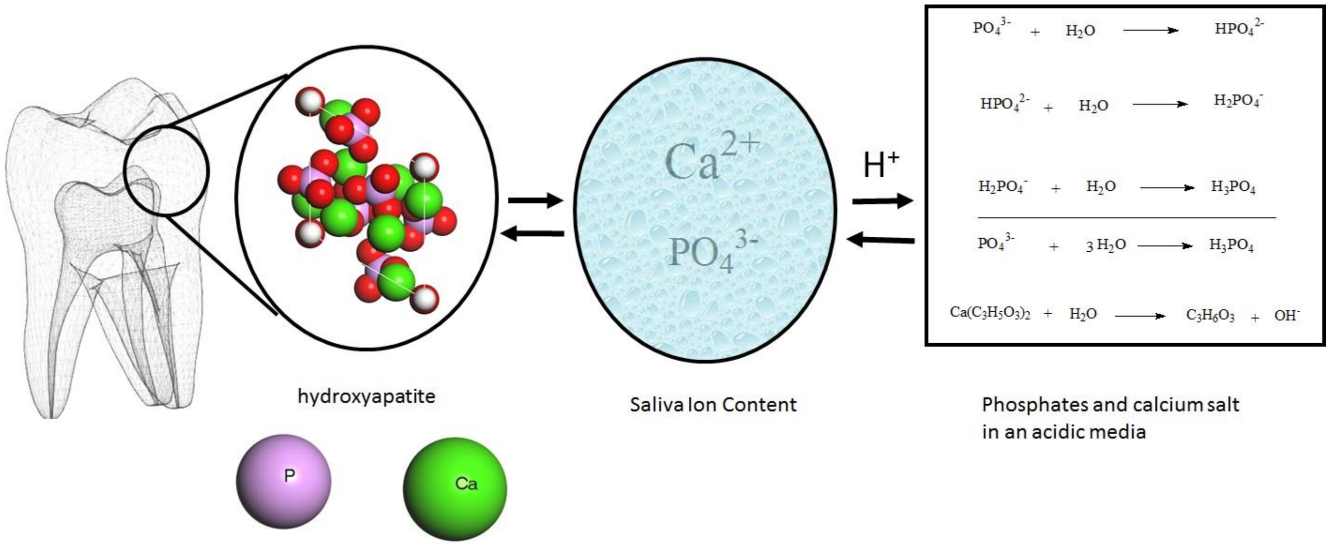

Figure 2- 3: Showing the effect of erosive agents in the cycle of demineralisation and remineralisation of enamel (adapted by the researcher).

2.2.1 Histopathology and Management of DH

As previously stated, dentin consists of microscopic dentinal tubules. Several studies (Addy 2000; Zero and Lussi 2005; Lussi 2006; Cummins 2010; Miglani, Aggarwal and Ahuja 2010; Borges, Barcellos and Gomes 2012) suggested that for DH to be initiated, the dentinal tubule must be exposed to the external environment, which is caused by the removal of the protective smear layer covering the tubule. Notably, SEM studies (Holland 1994; Lee *et al.* 2007; Chivu-Garip *et al.* 2012; Davari, Ataei and Assarzadeh 2013; Moura *et al.* 2016) have shown that tubules in exfoliated teeth (sensitive tooth) are eight times more numerous, twice larger in diameter and are open, whereas tubules in non-sensitive tooth are less numerous, smaller, and usually blocked.

Understanding how DH is initiated is therefore imperative to develop an effective strategy to mitigate and manage its onset. For example, many authors (Miglani, Aggarwal and Ahuja 2010; Gillam 2015; John *et al.* 2015; Yu *et al.* 2016) have postulated a number of theories such as direct innervation theory, odontoblast receptor theory and hydrodynamic theory to explain the mechanism of DH. Low, Allen and Kontogiorgos (2015), however, argued that the direct innervation theory and odontoblast receptor theory are not widely considered. Currently, the hydrodynamic theory is most extensively used to explain the mechanism of DH. Hence, and as highlighted in Chapter three Section 3.3, this study is premise on the principle of the hydrodynamic theory that any decrease in the dentinal fluid movement should result in the reduction of DH (Pereira, Segala and Gillam 2005). Thus, and as observed by Yu *et al.* (2016), the most effective strategy in the management of DH is to effectively occlude the dentinal tubules to prevent fluid flow. Bearing these in mind, Yang *et al.* (2016) advocated using a desensitising paste to physically block the exposed dentine tubules.

2.2.2 Occluding and nerve depolarising agents in DH Management

Traditionally, the first line of 'at-home' treatment is to use over the counter products (OTC) in the form of desensitising toothpaste (Low, Allen and Kontogiorgos 2015; Arnold *et al.* 2016). Saeki *et al.* (2016) noted that these OTC products contain active ingredients that either desensitise the nerve tissue at the base of the dentin tubule or function by promoting dentin tubule occlusion. Over the last decades, the oral care industry has witnessed the proliferation of different occlusion materials that claim to occlude open dentin tubules (Arnold, Prange and Naumova 2015). Studies by Aranha, Pimenta and Marchi (2009), Chivu-Garip *et al.* (2012), and Suri *et al.* (2016), however, noted that most commercial desensitising agents are unable to provide instant or longtime relief. This is critical as it is generally recognised that long time relief is a highly motivating factor for DH patients (Schmidlin and Sahrmann 2013), and thus, complete and robust dentin occlusion is the most promising treatment strategy to achieving relief.

Miglani, Aggarwal and Ahuja (2010), and Chivu-Garip *et al.* (2012) therefore clarified that desensitising agents ideally need to be non-irritant to the pulp, relatively painless on the application, easily applied, rapid action, effective for a long period, without staining effects and consistently effective. The advice of the aforementioned authors will be considered when developing EB@TiO_2 desensitising toothpaste. It is noted that the action of desensitising paste is dependent upon the active ingredients and cognisance is taken of the effect of these ingredients will cause on the oral cavity.

Literature (Panagakos, Schiff and Guignon 2009; Schiff *et al.* 2009; Cummins 2010; Shiau 2012; Addy and West 2013; John *et al.* 2015) have documented occluding agents such as (calcium carbonates; stannous fluoride; arginine and calcium carbonates, nano-hydroxyapatite; strontium salts, fluoride; oxalates; bioglass; portland cement) and nerve interfering product (potassium salt), which are delivered through desensitising paste. Although most commercial desensitising toothpaste listed in Table 2-3 claim to occlude open dentine tubules, Arnold, Prange and Naumova (2015) contend that this occlusion is superficial and may be dissolved with acids. According to their report, the occlusion abilities of these pastes are easily reopened under erosive attack (Arnold, Prange and Naumova 2015). The authors concluded that the effectiveness of desensitising toothpaste should be in their active ingredients, and thus, are not complete in any of the toothpaste highlighted in Table 2-3.

Moreover, potassium salt containing desensitising toothpaste are thought to diffuse inside the dentin tubules and lower the excitability of the pulpal nerve fibers. Pashley and Matthews (1993), and Pashley *et al.* (2002) moots that the flow of dentinal fluid is outward from the pulp towards the tooth surface, which invariably obstructs diffusion towards the pulp. Similarly, strontium salts containing desensitising toothpaste was thought to occlude dentine tubules by forming strontium apatite, which could be absorbed onto the connective tissue of the dentin (Gedalia *et al.* 1978). Arnold, Prange and Naumova (2015), however, contend that dentine tubule occlusion by strontium salts has not yet been proven. Their assertion supports other authors (Pearce, Addy and Newcombe 1994; Silverman *et al.* 1996) who only observed the reduction of pain perception in patients who used strontium salts.

With reference to the use of arginine and carbonates as desensitising agents, studies by Petrou *et al.* (2009), and Lavender *et al.* (2010) have demonstrated that arginine combined with calcium carbonate desensitising toothpaste occlude dentine tubules by converting calcium deposits to calcium phosphate. Arnold, Prange and Naumova (2015), however, stressed that calcium phosphates are soluble in acidic environments which subsequently become unstable upon dietary acid challenge. This is concerning, particularly in light of several randomised controlled clinical trials that demonstrated arginine and calcium carbonate toothpaste effectiveness for only 8 weeks after treatment (Ayad *et al.* 2009; Docimo *et al.* 2009; Schiff *et al.* 2009; Fu *et al.* 2010; Que *et al.* 2010). More so, the early work of Greenhill and Pashley (1981) reported that oxalates containing desensitising toothpaste form oxalate crystals within the dentin tubules, which act as desensitising agent. A systematic review conducted by Cunha-Cruz *et al.* (2011) regarding the effectiveness of oxalates in the treatment of DH concluded that oxalates had no effect on DH when compared against the placebos. Their reports support the early work of Banfield and Addy (2004) who noted that desensitising pastes containing oxalates or calcium phosphate are acid labile and their occluding ability may easily be removed.

Table 2- 3: Common desensitising tooth paste active ingredients

Product name	Active ingredient	Company
Elmex Sensitive Professional	Pro-Argin, calcium carbonate	GABA
Sensodyne Rapid	Strontium acetate	Glaxo Smith Kline
Sensodyne Repair	Stannous fluoride	Glaxo Smith Kline
BioRepair	Zinc-carbonate hydroxyapatite	Dr. K. Wolff
Colgate Total Sensitive	New silica	Colgate-Palmolive
Dontodent Sensitive	Tetrapotassium pyrophosphate, hydroxyapatite	DM Dogeriemarkt

Source: Arnold, Prange and Naumova (2015)

Additionally, Arnold, Prange and Naumova (2015) noted that most commercially available toothpaste contains fluoride. Although fluorides like other occluding desensitising agents, may block the dentine tubules, Arnold, Prange and Naumova (2015) point to the fact that there is high incidence of DH amongst the general population negates the action of fluoride. They argued that the mechanisms of the action of fluorides in the treatment of DH remains unclear. In contrast to some other studies (Naumova *et al.* 2010) that speculated the ability of fluoride-containing pastes in enhancing mineralisation of hydroxyapatites, Arnold, Prange and Naumova (2015) stressed that the enhancement of fluoride in the formation of hydroxyapatite is yet to be demonstrated.

Given the above drawbacks of many OTC desensitising pastes to effectively managed DH, literature (Kovtun *et al.* 2012; Tian *et al.* 2014; Wang *et al.* 2014) have assumed that a novel approach in the treatment and management of DH should be the use of various combinations of nanoparticles. According to Arnold, Prange and Naumova (2015), the idea behind this approach is that nanoparticles can easily penetrate into dentin tubules, which could act as mineralising agents that block fluid movement within the dentin tubules when combined with various agents. Although there are yet to be established gold standard treatment modalities in the management of DH (Schmidlin and Sahrmann 2013), the use of tubule blocking agents is a growing area of interest in the healthcare industry as an effective strategy for the management of DH (Tian *et al.* 2014). The advocacy for the use of nanomaterials in the management of DH will lead to the departure for the next section.

2.3 Nanomaterials in DH management

In recent years, Nanotechnology particularly as it applies to dentistry "Nanodentistry" has increasingly become a fascinating area of discourse for many researchers. For instance, Seth and Khan (2017) explicitly noted that the nanodentistry has the power to completely revolutionise the field of dentistry. The authors predicted that through nanomaterials and manipulating materials at the nanoscale level, painful procedures would become history as nanomaterials could reduce painfully dental procedures, help remineralise the tooth and associated structures and help maintain oral hygiene. This section illustrates the evidence base for the application of nanomaterials in occluded dentin tubules.

Nanomaterials can be classified according to their dimensions (zero-dimensional, one-dimensional, two-dimensional, and three dimensional) or their structures (Nanopores, Nano tubes, quantum dots, Nanoshells, dendrimers, liposomes, Nano rods, fullerenes, Nanospheres, Nanowires, Nanoballs, Nanorings, Nanocap etc.). Li *et al.* (2013) revealed that nanomaterials have a small size, larger surface area, high surface energy, and large proportional of surface atoms. Owing to these unique properties, literature (Besinis *et al.* 2015; Samiei *et al.* 2016) documented that nanoscale materials have attracted interest as a more efficient method of delivering antimicrobials and remineralisation agents into dentinal tubules (Figure 2-4). The size and reactivity of nanoparticles (NPs) may allow them to be delivered further into dentinal tubules, with an enhanced potential for decontamination, remineralisation and reduced sensitisation compared with contemporary treatment regimens (Besinis *et al.* 2015). In addition, the solubility and reactivity of NPs are significantly increased because of their high surface energy and a large surface (de Villiers, Aramwit and Kwon 2009). More so, the large surface area of NPs also provides a high affinity and allows them to easily deposit on irregular spaces (Lee, Kwon and Kim 2008).

Significantly, Tian and his co-authors speculate that due to the superior dispersion of nanomaterials, it can easily enter dentinal tubules of 2–3 µm (Tian *et al.* 2014). The above claim by the aforementioned authors strongly reinforced the superiority of nanomaterials as a prime candidate for dentinal tubule occlusion.

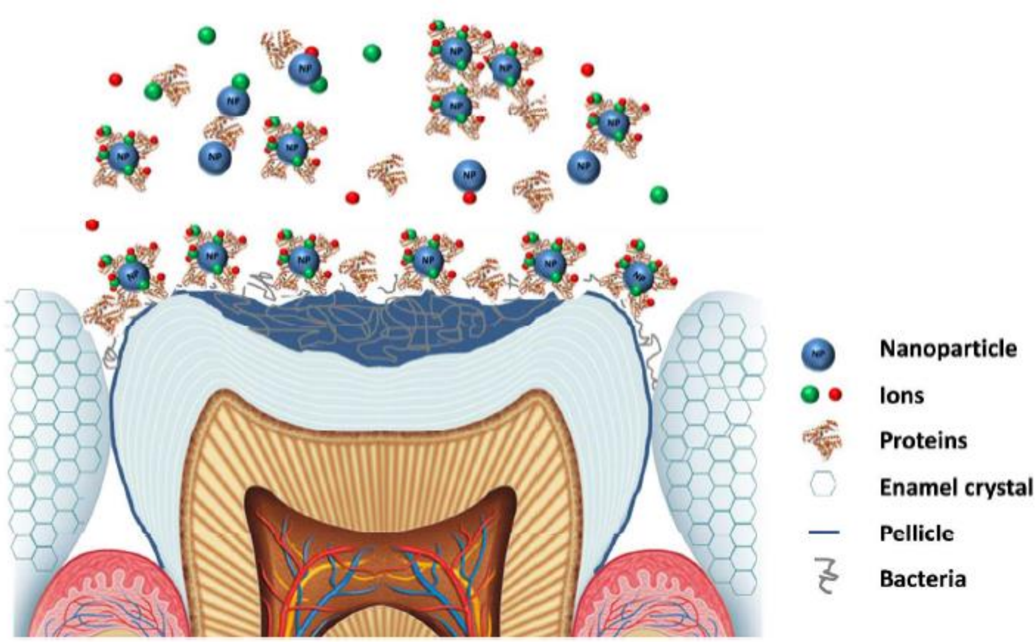

Figure 2- 4: Nanoparticles interaction with tooth surface (Source (Besinis et al. 2015)).

2.3.1 Mesoporous silica nanoparticles

In recent time, mesoporous silica nanoparticles (MSNs) have shown promising results as a biomaterial attributed to their stable network structure, large surface area, adsorption performance, thermal, and chemical stability (Fu *et al.* 2013). In terms of the effectiveness of MSNs in occluding dentin tubules, and as highlighted in Table 2-4, several studies have been carried out to establish its efficacy as an occluded material either alone or in the modified form with other nanomaterials.

Table 2- 4: Recent studies on MSN occluding abilities

Authors	Particles size	Other materials	Type of study	Model
(Tian *et al.* 2014)	50-80nm	Ca^{2+} and PO_4^{3-}	*In vitro study*	Human third molars
(Chiang *et al.* 2014)	N/A	Calcium carbonates	*In vivo /In vitro study*	Animal
(Yu *et al.* 2016)	50 nm	Nanohydroxyapatite	*In vitro study*	Human third molars

Tian *et al.* (2014) in their studies reported on the dentinal tubules occluding abilities of MSNs and or calcium or phosphate modified MSN (Ca^{2+}@MSN and PO_4^{3-}@ MSN). The authors concluded that MSNs or its modification exhibited a superior occluding ability. It was demonstrated that MSN or Ca^{2+}@MSN and PO_4^{3-}@ MSN could effectively occlude dentinal tubules at both the exterior open end of dentinal tubules and in the depth of dentinal tubules. Chiang and his co-authors concluded that calcium carbonates ($CaCO_3$) containing MSN mixed with 30% calcium triphosphate (H_3PO_4) effectively occluded dentin tubules (Chiang *et al.* 2014). In a different study, Yu *et al.* (2016) demonstrated that a novel biocomposite based on the medication of nanohydroxyapatite and mesoporous silica nanoparticles (nHAp@MSN) were highly efficient in occluding dentinal tubule with the occlusion showing a high acid-resistant stability. The authors attributed the acid resistant stability of the biocomposite to the unique acid resistance of mesoporous silica. Nevertheless, they noted that intratubular occlusion in the samples treated with MSNs alone was inferior compared to the combination of nHAp@MSN; which they claimed could by washed away be a citric acid solution or deionised water. This, they pointed out could be attributed to the particle size differences between the MSNs and nHAp@MSN. More so, it was surmised that the presence of nHAp in the biocomposite may lead to favourable blockage insider the tubules which could offer a protective effect against acid attack.

Drawing from the above, it is not surprising to note that the synergetic effect of the various nanomaterials reported offers in-depth occlusion and acid resistant stability. This goes a long way in supporting the proposition made by other scholars (Kovtun *et al.* 2012; Wang *et al.* 2014) that the combination of nanomaterials offers a better treatment strategy for the management of DH.

2.3.2 Nano hydroxyapatites crystals

Nanohydroxyapatite (nHAp) is one of the main structure of the dental hard tissues expressed chemically as $Ca_{10}(PO4)_6(OH)_2$. According to Hannig and Hannig (2010), the biocompatible and bioactive nature of nanohydroxyatite material has endeared its acceptance in medicine and dentistry. In dentistry, for example, nanohydroxyapatite has been extensively investigated for its remineralisation potentials (Table 2-5). For instance, Ohta *et al.* (2007) observed the surface of dentin specimens treated with nanohydroxyapatite using Field Emission Scanning Electron Microscope (FESEM). Their study revealed that nano-HAp uniformly occluded the dentinal tubules with a dentinal plug and a protective layer on the surface of the dentine was also formed. Similarly, Tschoppe and his colleague investigated the in vitro effects of nanohydroxyapatites (nHAp) toothpastes on remineralisation of bovine tooth (Tschoppe *et al.* 2011). They observed an increased in remineralisation of dentin and enamel with toothpastes containing nHAp compared with amine fluoride toothpastes. Due to the positive occlusion notice in the study, the authors reasoned that nHAp can promote remineralisation.

Furthermore, and in a separate study, Al-maliky *et al.* (2014) reported on the occluded potentials of nHAp with or without laser CO_2 treatment. The authors concluded that the combination of nHAp paste and a CO_2 laser of moderate power density occluded the dentinal tubules and reduced the permeability of exposed dentin. In addition, Beglar and his team revealed that pure nHAp as well as combination of nHAp in 1%, 2%, and 3% fluoride exhibited strong resistance to degradation. The authors found that nHAp together with the doped fluoride were strongly effective in covering the dentin surface and also showed plugging effects on the tubules (Baglar *et al.* 2018).

The above mentioned effectiveness of nHAp in the management of DH is further supported by numerous clinical studies (Browning, Cho and Deschepper 2012; Vano *et al.* 2014; Low, Allen and Kontogiorgos 2015; Vano *et al.* 2018). Browning and his team investigated the effect nHAp paste on bleaching-related tooth sensitivity (Browning, Cho and Deschepper 2012). The authors observe that the use of a toothpaste containing nHAp can decrease tooth sensitivity in individuals undergoing bleaching without any desensitising agent. Equally, Vano and co-authors reported that nHAp provides quick relief from dentin related sensitivity symptoms which was higher than fluoride containing toothpaste (Vano *et al.* 2014).

In another clinical study, Low, Allen and Kontogiorgos (2015) assessed the ability of a toothpaste containing nHAp together with potassium nitrate, sodium monoflurophoshate, antioxidants phloretin, ferulic and silymarin in reducing tooth pain associated with DH measured using a visual analog scale (VAS). The authors speculate that when applied daily, the synergetic effect of nHAp and other mentioned constituents can significantly and quickly reduce DH. It was also found that the outstanding results of speed and effectiveness of the toothpaste were related to the activity of nHAp as well as the antioxidants. In a more recent clinical study, Vano and his team objectively (airblast and tactile test) and subjectively (VAS) measured the effectiveness of nHAp to occlude dentin tubules. The authors found that that the application of 2% nano-hydroxyapatite was an effective desensitising agent providing relief from symptoms after 2 and 4 weeks (Vano *et al.* 2018).

In light of the positive reports from the use of nHAp in dentin treatment, the early prediction of Khetawat and Lodha (2015) that in near future new products containing nHAp would be a breakthrough in the treatment of dentinal hypersensitivity resonates further. This, and according to Besinis, van Noort and Martin (2012) may be related to the high biological activity and reactivity of nHAp which enable it to bind to dentin apatite and infiltrate the dentinal tubules.

Table 2- 5: Recent studies on nanohydroxyapatites occluding abilities

Authors	Type of study	Model
Ohta *et al.* (2007)	*In vitro study*	Human molars
Tschoppe *et al.* (2011)	*In vitro study*	Bovine incisors
Browning, Cho and Deschepper (2012)	Clinical	Humans
Vano *et al.* (2014)	Clinical trial	Humans
Al-maliky *et al.* (2014)	*In vitro study*	Human molars
Low, Allen and Kontogiorgos (2015)	Clinical trial	Humans
Vano *et al.* (2018)	Clinical trial	Humans
Baglar *et al.* (2018)	*In vitro study*	Human molars

From the above, it is imperative to note that nano dentistry through the modification and manipulation of nanomaterials presents a potent technology for better and improved oral health care services. This new technology appears to be highly effective in the management of pain associated with dentin hypersensitivity (de Melo Alencar *et al.* 2019). However, these materials are traditionally synthesised with the use of toxic or noxious compounds, which may limit their biomedical applications (Hua *et al.* 2017). Moreover, the environmental concern and cost of production seems to overwhelm the benefits associated with the use of these materials (Duan, Wang and Li 2015). Hence, new technology and method of synthesising and modifying the said materials for the medical and dental application become highly desirable.

2.4 New approach to DH management

Interestingly, studies (Nakashima *et al.* 2009; Teo, Ashley and Louca 2014; Rahardjo *et al.* 2015) have suggested that desensitising paste containing nano-sized calcium has the potential to remineralise damaged teeth due to its unique properties. They propounded that nano-sized calcium is retained on oral surfaces, and subsequently released calcium ions into oral fluids. More so, Nakashima *et al.* (2009) argued that nano-sized calcium carbonates surface area would be extremely large due to its great size when compared against calcium carbonates powders for toothpaste abrasives. As a consequence, the rate of dissolution of nano-sized calcium carbonates will be faster, which can be highly effective in remineralising damaged teeth. The aforementioned work, therefore, suggests that developing a new EB@TiO_2 desensitising paste using nano-sized eggshell is important in the treatment and management of DH. Equally important, authors (Tombak and Madejski 2006; Henuset 2011; King'Ori 2011) have maintained that the calcium of eggshell is similar to bone and teeth, hence it is used in certain toothpaste as an abrasive cleaner for dental plaque. The characteristics and chemical composition of eggshell will serve as the point for departure for the next section.

2.4.1 Composition and characteristics of eggshells and titanium dioxide

2.4.1.1 Overview of eggshell structure, properties, and applications

Generally, and as highlighted by several studies (Giron 2002; Campos *et al.* 2004; Cardoso *et al.* 2005), eggshells are lightweight, crushable, texturally hard and porous to protect the chicken embryo from microorganisms. Freire and Holanda (2006), and Onwubu (2016) emphasised that the porousness of eggshells enables it to be crushed into various particle sizes ranging from 2 to 900 microns. Several other authors (Tsai *et al.* 2008b; Baláž *et al.* 2015; Baláž, Ficeriová and Briančin 2016; Nasrollahzadeh, Sajadi and Hatamifard 2016; Baláž 2018) have advised that through the application of appropriate experimental conditions it is possible to reduce the particles of eggshells into finer micron and nano-sizes. This is pertinent to the experimental work of this study. Consequently, the eggshell powder produced could potentially be used for various applications (Baláž 2018).

Furthermore, Hincke *et al.* (2012) revealed that the avian eggshell epitomises the most advanced amniotic egg in oviparous vertebrates. Importantly, and according to the above authors, avian eggshells function to regulate the exchange of metabolic gases and water whilst protecting the contents of the egg from microbes and the physical environment (Hincke *et al.* 2012). They also reveals that many physiological, biochemical, structural and morphological studies conducted on avian eggs used domestic chicken eggs or *Gallus gallus*. Unlike other avian shells such as ostrich, duck, goose, and elephant bird, chicken eggs are abundantly available and renewable (Hincke *et al.* 2012). Hence, the foregoing section highlights the considerable body of research dealing with the domestic chicken eggshell, which has provided insight into its structure and properties.

As illustrated in Figure 2-5, Chien, Hincke and McKee (2009), and Kang *et al.* (2010) suggest that the chicken eggshell has a well-defined structure consisting of the: mammillary body layer; thick palisade layer; and transitional vertical crystal layer. A non-calcified cuticle layer coats the eggshell. The earlier work of Dennis *et al.* (1996) noted that the transitional, inner zone of the eggshell cuticle contains spherical aggregates of hydroxyapatite.

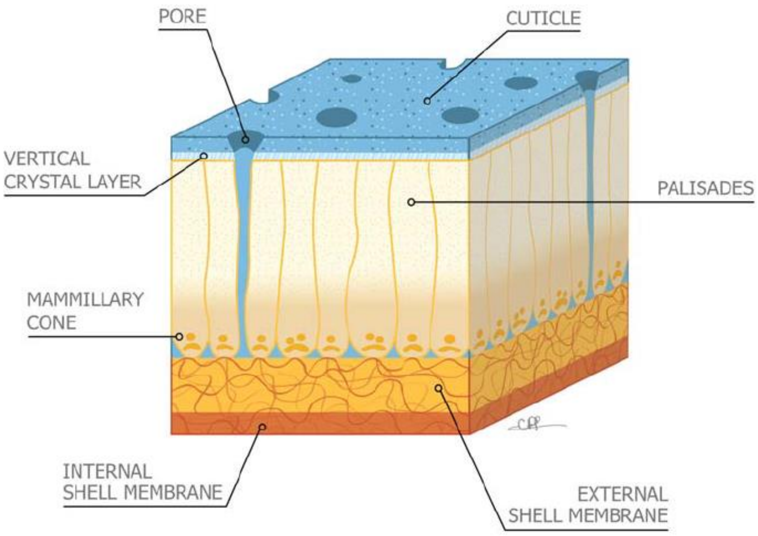

Figure 2- 5: Eggshell structure (Source: Hincke et al. (2012))

More so, Stadelman (2000), Hincke *et al.* (2012), and Baláž *et al.* (2015) have reported that chicken eggshell is a natural bioceramic composite composed of organic and inorganic compounds. Along with its membranes, eggshells are composed ~ 95% of calcium carbonates in the form of calcite and ~ 5% of type X collagen, sulphated polysaccharides (Stadelman 2000). Other components include: Magnesium carbonates ($MgCO_3$); 1% apatite (Ca_3 $(PO4)_2$ 1% and organic matter (4%) (Stadelman 2000). Despite the unique chemical composition of chicken eggshells and its potential use in medical applications, they have not extensively gained attention for its medical benefits (Murakami *et al.* 2007). This next section gives an insight into the current medical and dental applications of eggshell.

2.4.1.2 Medical and dental applications of eggshell.

In the last decade, eggshells have been limited in used as an additive for animal feed and human nutrition (Cordeiro and Hincke 2011), coating pigment for inkjet printing

(Hsieh 2009; Yoo *et al.* 2009), dye-effluents remover (Slimani *et al.* 2014), and as bio-fillers for polymer-based composites (Mohan and Kanny 2018; Pajtášová *et al.* 2018). Recently, there has been increased interest in the use of eggshell and its associated membrane for a variety of medical applications. For example, Abdulrahman *et al.* (2014) demonstrated how calcium carbonate derived from eggshell could be used to produce hydroxyapatite, which is mainly used in bone and dental treatments. They argued that eggshell based hydroxyapatite and nanohydroxyapatite could reduce the cost of treatment in bone repair or replacement. Resonating with them, Khandelwal and Prakash (2016) elaborated that the composition and properties of eggshells have supported its use in the synthesis of hydroxyapatite and nano-hydroxyapatite which shares characteristics with the natural building blocks of enamel.

Moreover, and as noted by Oliveira, Benelli and Amante (2013), the use of eggshell as an alternative source of calcium carbonate may alleviate the impact on the natural reserves of limestone, which is a non-renewable natural source of calcium carbonate (Boron 2004). A noteworthy point is that calcium carbonate is a rich source of mineral for the pharmaceutical industry, which is a base material for developing medicinal and dental preparations. Murakami *et al.* (2007) noted that calcium carbonate from eggshell has great potential in the development of alternative ingredients for medical applications such as food additive, dental formulations, calcium supplement, and as a component for bone implants. They also elaborated that the dearth of information on the medical application of eggshell suggests that there is a promising area worth exploring.

From a dental and orthopaedic surgery context, eggshell is an adaptive innovative bone graft substitute that has shown favourable results in the treatment of bone and dental defects. For instance, Macha *et al.* (2015) outlined that eggshells have been used in biomedical applications directly as a bone substitute either for maxilla-facial surgery or as sources of hydroxyapatite in bioceramics productions. They noted that the progress recently achieved in orthopaedic and dental applications has increased the demand for eggshell natural hydroxyapatites bioceramics. For example, Kattimani *et al.* (2014) reported that eggshell showed early bone regeneration and values of density measurements equal or more than that of surrounding normal bone. Their work supports other studies (Kim *et al.* 2008; Lee *et al.* 2012) were eggshell was highly effective in the treatment of bone defects in rabbit graft models. Similarly, Kattimani *et al.* (2014) reported that eggshell showed complete bone formation in the treatment of human maxillary cystic bone defects. The aforementioned studies, therefore, provide evidence that eggshell was the gold standards in terms of bone regeneration.

In addition, and as revealed by Hassan (2015) chicken eggshell has a calcium content of approximately 38%, which offers a promising but little utilised source of calcium for the human supplement. He elaborated that calcium derived from eggshells is 90% absorbable in the human body. This aligns with the early work of Oguido, de Angelis and Yada (1995). They noted that the absorption of calcium from eggshell is greater in males when compared with the absorption of calcium found in milk. The above authors highlight the potential benefits of eggshell in the treatment of bone disease associated with calcium deficiency.

Drawing from the above applications of eggshell, it can be gathered that eggshell has the potential for the remineralisation of damaged teeth. It should be interesting to note that eggshells are currently being investigated for their remineralisation capabilities (Macri 2016). This corroborates further with the work of Mony *et al.* (2015), and Haghgoo *et al.* (2016) that eggshell powder solution was effective on early enamel carious lesions. As argued by the said authors, the high pH of eggshell coupled with the rich bioavailable calcium content of eggshell favoured the remineralisation of damaged teeth (Mony *et al.* 2015; Haghgoo *et al.* 2016). In view of achieving optimum remineralisation and robust treatment of DH, the aforementioned high calcium content found in an eggshell is pertinent to this study.

2.4.2 Characteristics and applications of titanium dioxide

According to García-Valverde *et al.* (2014), naturally occurring titanium dioxide exists in different forms, namely: anatase, rutile, and brookite that differ in their crystalline structure. Notwithstanding the different forms, García-Valverde *et al.* (2014) emphasised that titanium dioxide is broadly used as precursors to synthesize TiO_2-structures at the nanoscale level where spherical particles and elongated materials are possible. They also noted that nano-sized titanium dioxide exhibits extraordinary characteristics such as inertness, chemical, and thermal stability, high refractive index, non-toxicity, low cost, durability, corrosion, and acid resistance. Essentially, these properties have supported its use as a pigment in paint, glazes, enamels, plastics, paper, fibers, foods, pharmaceuticals, cosmetics, and toothpaste (Diebold 2003;

Eastern Research Group 2010; Mital and Manoj 2011; Weir *et al.* 2012; Fu *et al.* 2014). Particularly, the earlier work of Diebold (2003) highlighted that titanium dioxide contributes to the biocompatibility of titanium bone implants. It can, therefore, be gathered that titanium can be used to develop a desensitising toothpaste in remineralising damaged teeth.

Significantly, Tao, He and Zhao (2015) suggested that the preparation and application of $CaCO_3$-TiO_2 core-shell nanocomposites particle materials would improve the acid resistance of calcium carbonates. More importantly, Lin, Dong and Jiang (2009), and Tao, He and Zhao (2015) have shown that the mechanical activation of nano-sized calcium carbonates together with titanium dioxide improved the absorption capacity of calcium carbonates. Their work is pertinent to the study, particularly in developing a new desensitising dental toothpaste using nano-sized eggshell and titanium dioxide (EB@TiO_2) desensitising toothpaste.

As stated above, titanium dioxide, particularly in their nano-sized, has been extensively used in industrial production as well as scientific, biological, and medical fields. Yuan *et al.* (2010) reveal that nano-sized titanium dioxide is under investigation as a useful tool in advanced imaging and nanotherapeutics. Equally important, a study by Szaciłowski *et al.* (2005) reported that nano-sized titanium dioxide is being evaluated for its potential application in photodynamic therapy. Similarly, Wiesenthal *et al.* (2011) reported that nano-sized titanium dioxide in combination with other nano-preparations are currently being investigated as a novel treatment for skin disease such as acne; vulgairis; recurrent condyloma accuminata; atopic dermatitis; hyper pigmented skin lesions; and other non-dermatologic diseases. In addition, studies by Yuan *et al.* (2010), and Montazer *et al.* (2011) also reported that nano-sized titanium dioxide showed antibacterial properties when examined under UV irradiation.

Further to the above, anatase TiO_2 has been reported to be an effective antimicrobial agents that is compatible with the human body or environment (Rana and Misra 2005; Rawat *et al.* 2007). In particular, and in a more recent study, it was demonstrated that titania reduces the ability of bacterial such as *Staphylococcus aureus* (*S. aureus*) to adhere to surfaces by rupturing their cell membrane (Depan and Misra 2014). Given that the metabolic activities of bacterial in the oral cavity contribute to enamel demineralisation and subsequently dental caries (Chenicheri *et al.* 2017), this study assumed that due to the presence of titania (TiO_2), EB@TiO_2 could also act as antimicrobial agents in the prevention of dental caries.

Moreover, it should also be mentioned that titanium dioxide is allowed as food additives (E171) at *"quantum satis"* in Europe, which means as much of the substance that is needed for the desired effect, but not more (Ropers *et al.* 2017).

2.4.2.1 Toxicological concerns of nano-sized titanium dioxide

Natarajan *et al.* (2014) reported that a high exposure rate of titanium dioxide causes health concerns in animal models. Similarly, Nel *et al.* (2006) pointed out that several concerns have emerged on the potential undesirable effects of nano-sized titanium dioxide properties, particularly its harmful interactions with biological systems and the environment. They, however, acknowledged that the toxicological profile of nano-sized titanium dioxide is not completely understood (Nel *et al.* 2006). As a consequence, Cho *et al.* (2010) advised that there should be an appropriate assessment of the risks for the general and occupational exposed populations requiring nano-sized titanium dioxide hazard identification, and dose-response data (Shi *et al.* 2013). According to the report, such risk assessment should include characterising the physicochemical properties of the nano-sized titanium such as the particle size, shape distribution, etc. The advice of the aforementioned authors will be considered when developing EB@TiO_2 desensitising toothpaste.

In light of the above, and in respect to the potential adverse health effects of nano-sized titanium dioxide, Kang *et al.* (2008) have clarified that nano-sized titanium dioxide, when used in low concentration, is biologically inactive and physiologically inert in both humans and animals, hence would pose no risks to humans. This is consistent with (Fartkhooni, Noori and Mohammadi 2016) who reported that the *in vivo* use of nano-sized titanium dioxide in small amounts in the medical fields does not cause any toxicity and disturbances in the body. The aforementioned authors support other studies (Hines 2010; National Institute for Occupational Safety and Health 2011) were pulmonary inflammation is observed in animals ingested with a sufficiently high dose of nano-sized titanium dioxide.

From the foregoing section, it can be gathered that the amount of titanium dioxide in consumer products may be critical to the associated health concerns. It also appears that there is a strong correlation with the amount of nano-sized titanium dioxide used and its associated toxicity. In order to characterise the cytotoxicity and toxicological profile of nano-sized titanium dioxide, this study will ensure that the developed EB@TiO_2 desensitising toothpaste is in compliant with ISO/TR 1321 in terms of the potential risks and safety concern of the use of nanomaterials.

Overall, and given the desirable properties of titanium dioxide (TiO_2) and the remineralisation potentials of eggshells (EB), a new EB@TiO_2 bio composite will be highly important for treating DH. The next section therefore examine the method of preparation of EB@TiO_2.

2.5 Mechanical preparation of eggshell and titanium (EB@TiO_2)

Traditional dependency on a solvent in most chemical reaction is becoming less viable and sustainable in the event of climate change facing the world (Clarke *et al.* 2018). In this aspect, mechanochemistry offers an advantageous prospect in composite material preparation. In fact, the modification of eggshell waste through ball-milling to obtain a novel material has gained numerous attention amongst researchers owing to their environmental friendliness, less use of harmful organic solvents and energy as well as its reproducibility with high yield under simple and easy operating conditions (Baláž *et al.* 2013; Baláž 2018). According to (Baláž 2018), ball-milling is a tool of mechanochemistry that combines solid-state approach and mechanical energy input for various desired applications. The author hinted that ball-milling natural material such as eggshells offers the prospect to change their applications potential to a new level (Baláž 2018). Significantly, Battistella *et al.* (2011) advocated for the use of biomaterial obtain from a natural source in dentistry due to their outstanding healing properties, biocompatibility with the natural tissues, abundant availability, and low cost.

2.5.1 The Science of Mechanochemistry

As revealed in the literature (Hua *et al.* 2017), mechanochemistry harnessed mechanical force to induce chemical reactions or structural changes of material that is similar to those provided by thermochemistry (energy by heat), photochemistry (energy by light) or electrochemistry (energy by electrical potential). Owing to these attributes, mechanochemistry is considered a powerful technique for modulation of chemical reactivity and preparation of materials with high performances (James *et al.* 2012; Brantley, Wiggins and Bielawski 2013). By this mechanical activation, it becomes possible not to only cause structural changes in the material but also reduce their particles sizes (Tsai *et al.* 2008a).

2.5.2 Mechanochemistry tools

Over the last decade, mechanochemical tools have transcendent from its earliest mortar and pestle (Fig 2-6A) to more elaborate and sophisticated electronic devices (Fig 2-6B). Among these new devices, the ball-milling, tumbling mill, planetary mill, mixer, and rolling mill, etc. (Fig 2-7) have been extensively utilised as a tool for a mechanochemical reaction. Ball-milling is a common comminution method of producing fine powder in many industrial fields. Baláž (2018) noted that ball-milling combines solid-state approach and mechanical energy input for various desired applications. It is worth mentioning that this study would be using the planetary ball-milling machine for the preparation of nanosized EB@TiO_2. Interestingly, the planetary ball-milling reduces particles into fine powders through the imposition of impact and friction forces (Tsai *et al.* 2008a).

The planetary ball mills by Fritsch company (invented 1961, Germany) are considered as the mainstays of many laboratories using the principles of mechanochemical reaction in their material preparations (Baláž *et al.* 2013). The attractiveness of the Fritsch product in the mechanochemical reaction is the durability and automation of the unit. For instance, the operator has the discretion to vary the setting, milling time, and speed to suit his or her desired applications (Fokina *et al.* 2004). More so, the Fritsch unit has an energy density about 100–1000 times higher when compared against the earliest conventional milling equipment (Fokina *et al.* 2004). In the planetary ball-milling, the reagents, and ball(s) are loaded together (Figure 2-8), and the jars spin counter-directionally to the spinning disc that they are mounted on (Howard, Cao and Browne 2018). To ensure the achievement of an optimally modified EB@TiO_2, the above-stated method of actions of the use of the planetary ball-milling will be highly considered and adhered when preparing EB@TiO_2.

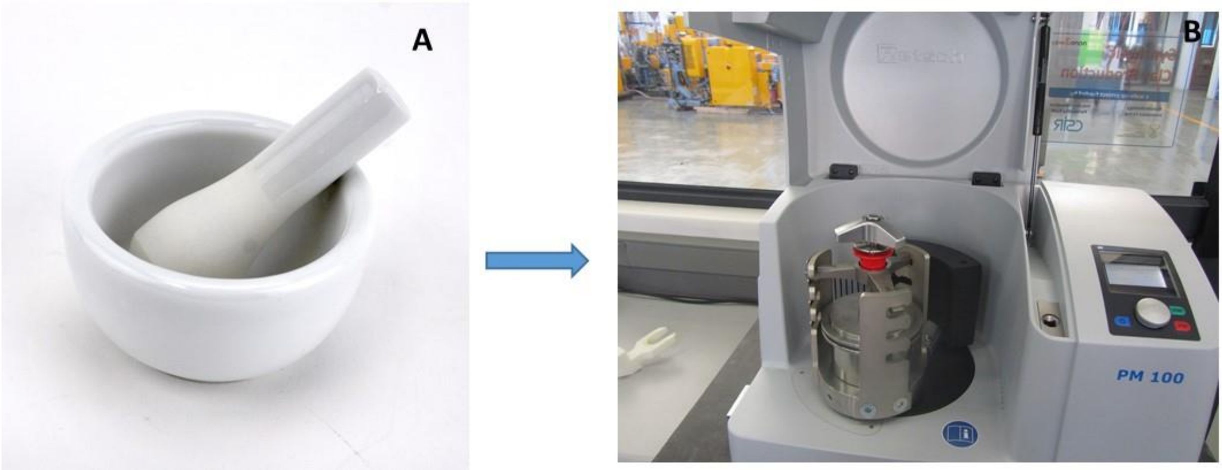

Figure 2- 6: Showing (A) mortar and pestle; (B) modern planetary ball-milling

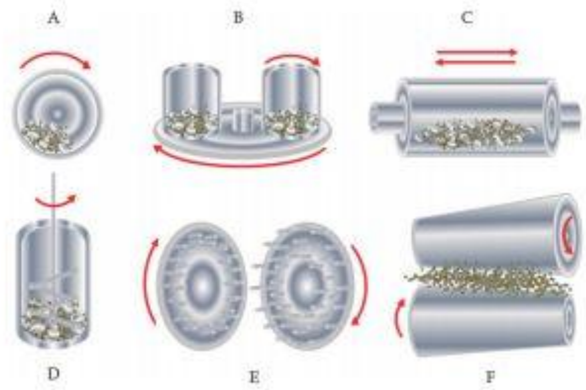

Figure 2- 7: Different types of mills for high energy milling: A – ball mill, B – planetary ball mill, C – vibration mill, D – attritor (stirring ball mill), E – pin mill, F – rolling mill (Source (Ferencz 2016))

The above mentioning devices requires high energy mills with a different working requirement such as compression, shear, impact, etc. It is worth stating here that several variables influence the milling process. As such, it is strongly recommended that the operator pay keen attention to parameters like the material of the milling media; the type of the mill to use; milling speed and time; ball-to-powder ratio, milling atmosphere; as well as the filling extent of the milling chamber (Baláž 2008).

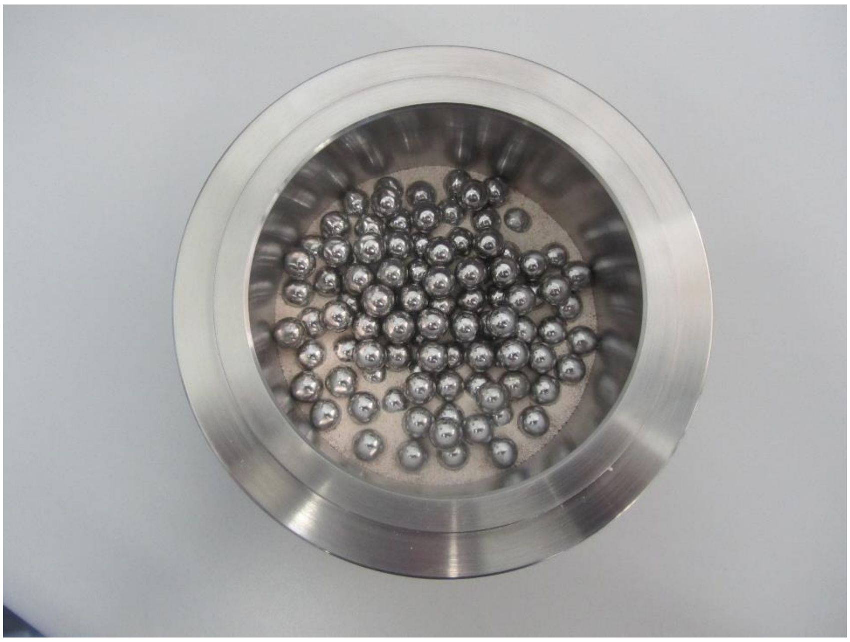

Figure 2- 8: Eggshell powder in a jar of 10mm balls

Apart from milling parameters, the type of mechanochemical vials or reactors and balls could also contribute to the end results (Baláž 2008). Table 2-6 highlights the different milling materials and their composition. It is critical to note that the use of these devices will depend on the intended applications and materials to be milled and cognisance of these in the preparation of EB@TiO_2 are hereby noted.

Table 2- 6: Material specification for grinding bowl and balls material

Materials	Use for grinding stock	Density in g/cm^3	Main components
Agate	Soft to medium-hard samples	2.65	(99.9% $SioO_2$)
Silicon nitride	Abrasive samples, metal-free grinding	3.25	(90% Si_3N_4)
Sintered corundum	Medium-hard, fibrous samples	3.9	(99.7% $A1_2O_3$)
Zirconium oxide	Fibrous, abrasive samples	5.7	(96.2% ZrO_2)
Hardened, stainless steel	Medium-hard, brittle samples	7.7	(16.0-18.0% Cr)
Tungsten carbide	Hard, abrasive samples	14.9	93% WC + 6% Co)

Source: FRITSCH (2006)

2.6 Requirement for Desensitising Toothpaste

According to Anusavice and Antonson (2013: 250) tooth cleaning substances such as dentifrices are available as toothpaste, gels, and powder. They point out that dentifrices have three essential functions, namely: (1) their abrasive and detergent actions provide more efficient removal of debris, plaque, and stained pellicle when compared with the use of toothbrush alone; (2) they polish teeth to provide increased light reflectance and superior esthetic appearance; and (3) they act as vehicles for the delivery of therapeutic agents such as desensitsing and remineralising agents. More so, the early work of Addy (2005) moots toothpaste has the potential to provide additional or adjunctive oral care benefits through chemical or physical interaction with the oral cavity.

In the last decade, studies (Addy and Hunter 2003; Lippert 2013; Arnold *et al.* 2016) have shown that toothpaste formulation have been manipulated to deliver chemical and physical mediated benefits ranging from the prevention of caries and supra-gingival plaque and calculus, the removal of extrinsic stains as well as the treatment of DH. While the therapeutic properties of toothpaste, particularly as it relates to the management of DH have been exhaustively explained in the sections above, the discussion now turns into the acid resistant requirement of toothpaste.

2.6.1 Acid resistant requirement

Enamel erosion has become a topical issue in recent years among clinicians and oral health care providers due to the increase in the consumption of acidic drinks such as soft drinks, energy drinks, and fruit drinks (Gambon, Brand and Veerman 2012). Previous studies (Lombardini *et al.* 2014; West *et al.* 2017) reported that these drinks have a pH (2-4) values that facilitate the dissolution of the tooth and the destruction of the mineral composition of the enamel. Several other authors (Harding *et al.* 2003; Luo *et al.* 2005) have demonstrated that as a result of the consumption of acidic drinks, the onset of caries and enamel erosion correlates positively together.

Whilst the tooth enamel is mainly composed of hydroxyapatite in the form of phosphate ions (PO_4^{3-}) and calcium ions (Ca^{2+}), Shellis, Featherstone and Lussi (2014), and Lussi and Carvalho (2015) argued that there exists an equilibrium between the tooth enamel crystals and the surrounding oral environment. The destabilisation of this equilibrium, particularly when the oral environment pH drops below a critical level (5.5 for enamel, and 6.2 for dentin), may result in the dissolution of tooth mineral composition in a process called demineralisation (Lussi and Jaeggi 2008). In contrast, the elevation of the oral environmental pH promoted by the natural buffer capacity of saliva, mineral gets reincorporated into the tooth through the process of remineralisation (Moron *et al.* 2013). While saliva is considered an important biological factor that dictates the intraoral neutralising effect of acid exposure, Neel *et al.* (2016) stressed that some medication and asthmatic inhalers may reduce the flow of saliva, thereby inducing Xerostomia. This, and according to Kargul *et al.* (1998), and Sivasitamparam *et al.* (2002), decreases the pH of saliva, and weakening its overall buffering effect against intrinsic and extrinsic acids. Hence preventive measures against enamel dissolution from acid and potentially permanent damaged should be a priority for oral health care providers (Lombardini *et al.* 2014; West *et al.* 2017).

Traditionally, toothpaste has been considered effective and accessible vehicles to improve enamel resistant against the erosive oral environment (Kato *et al.* 2010). In recent years, different ingredients have been added to the composition of toothpaste to enhance its protective effect against erosive substances. Amongst these ingredients, the use of topical fluoride to modify the effects of erosion at the tooth surface is well documented (Barbour *et al.* 2006; Almosa *et al.* 2013; Ganss, Schulze and Schlueter 2013; Krithikadatta *et al.* 2013). However, Moron *et al.* (2013) stressed that conventional fluoride containing toothpaste lack the capacity to protect sufficiently well against erosive substance.

Furthermore, Larsen and Richards (2002) revealed that at below pH 3 the protective effect of fluoride is diminished. Consequently, it has been suggested that the beneficial health effects against erosive attacks can be improved with the addition of calcium or calcium-containing material into toothpaste (Joiner *et al.* 2009). In line with the aforementioned author suggestion, and in view of the high calcium content in eggshell powder (Onwubu 2016), it is envisaged that EB@TiO_2 will provide protection to the tooth enamel against erosive substance. Equally relevant, it is stated above that the abrasives in toothpaste typically function as a stain and debris removal. Given the importance of toothpaste abrasiveness on the health of the enamel and dentin (Arnold *et al.* 2016), it becomes highly vital to examine the abrasive requirement of EB@TiO_2.

2.6.2 Abrasive requirement

Importantly, toothpaste contain abrasives that aid tooth cleaning and whitening, flavors (for fresh breath) and colorant to enhance their visual appeal (Lippert 2013). Anusavice and Antonson (2013) have broadly typified the various components of some desensitising toothpaste (Table 2-7). Lippert (2013) noted that abrasives are the most traditional toothpaste excipient and contribute secondarily to toothpaste rheology.

Table 2- 7: Typical Dentifrice components and compositions

Component	Composition (wt %)		Materials	Purpose
	Pastes/Gels	*Powders*		
Abrasive	20-55	90-98	Calcium carbonates. Dibasic calcium phosphate dihydrate. Hydrated silica. Sodium bicarbonate.	Removes plaque or stain. Polish tooth surface
Detergent	1-2	1-6	Sodium lauryl sulfate	Aids in debris removal, foaming, stability, solubiliser, anti-microbial, plaque inhibitory, and mouth feel.
Colourants	1-2	1-2	Food colourants	Are used for appearance
Flavouring	1-2	1-2	Oils of spearmint, peppermint, wintergreen, or cinnamon	Provides taste, feel, and freshness.
Humectant	20-35	0	Sorbitol, glycerine	Maintains moisture content, and flowability.
Water	15-25	0	Deionised water	Acts as suspension agent
Binder	3	0	Carrageenan	As thickener, prevents liquid-solid separation.
Fluoride	0-1	0	Sodium monofluorophosphate. Sodium fluoride. Stannous fluoride.	Prevents dental caries.
Tartar control agents	0-1	0	Disodium pyrophosphate. Tetrasodium pyrophosphate. Tetrapotassium pyrophosphate.	Inhibits formation of calculus above the gingival margin.
Desensitising agents	0-5	0	Potassium nitrate. Strontium chloride	Promotes occlusion of dentinal tubules.

Source: Anusavice and Antonson (2013)

Critically, and from a clinical perspective, Hara and Turssi (2017) moot that the abrasiveness of toothpaste be investigated for their safety concerns owing to the deleterious effects some may cause when brushing the tooth. Ideally, abrasive particles are harder than the stain but softer than sound enamel, hence stain can be removed without causing significant damage to the tooth surface (Joiner 2010). This is of utmost importance as excessive abrasive material could abrade tooth surface away, resulting in undesirable tooth wear (Hara and Turssi 2017).

According to Lippert (2013), the abrasive cleaning process is affected by various key parameters, such as particle hardness; shape; size; size distribution; concentration of its particles; and applied load during brushing. In light of these, toothpaste manufacturers often utilised calcium carbonate, dicalcium phosphate, silica, alumina oxide, calcium pyrophosphate, sodium metaphosphate, perlite, nano-hydroxyapatite, and sodium bicarbonate, etc. to provide an abrasive action (Lippert 2013; Hara and Turssi 2017). Concerning, and as highlighted by Field (2012), the action of said materials are, however, complex and chemically identical components may result in different abrasion characteristics.

Particularly, early studies (Absi, Addy and Adams 1992; Dyer, Addy and Newcombe 2000) have shown that the abrasivity of toothpaste could significantly influence the rate of abrasion than the features of the brush alone. Added to these, it has been reported that modern toothpaste due to their complex formulation has different relative abrasivity values (Field 2012). Hence an understanding of their clinical applications, effectiveness, and safety concern is highly paramount to prevent surface loss in both enamel and dentin (Hara *et al.* 2009; Hara and Turssi 2017). Of critical importance, Anusavice and Antonson (2013) reported that the abrasiveness of toothpaste may adversely impact on the exposed cementum and dentine surfaces.

Equally worrisome, Bizhang *et al.* (2016) reported that toothpaste with high relative dentin abrasivity values resulted in greater losses of dentin. Corroborating further, Arnold *et al.* (2016) reported that the abrasivity of toothpaste have an adverse effect on the occlusion of dentine tubules which could reopen the tubules during brushing procedure. Moreover, Addy (2005) caution that the abrasivity of toothpaste products have the potential to cause tooth wear, which could manifest DH arising from the exposed dentin. This is concerning as there is a strong correlation between the abrasiveness of the toothpaste, tooth brushing, and the occurrence of acute trauma, caries and periodontal disease, and dentine abrasion (Nunn 1996; Addy 2005; Field 2012). Noting these concerns, (Anusavice and Antonson 2013) cautioned that toothpaste does not have to be highly abrasive to clean teeth effectively. They pointed out that exposed root-surface cementum and dentine, respectively, are abraded at rates of 35 and 25 times the rate of enamel. Hence, the ideal toothpaste should provide the greatest possible cleaning action on tooth surfaces with the lowest possible abrasion rate (Anusavice and Antonson 2013). The advice given by the aforementioned authors is pertinent to this study, particularly in light of developing a new desensitising paste that will not cause adverse harm to the exposed dentin and healthy enamel.

Essentially, the 'holy grail' for manufacturers of abrasives and toothpaste manufacturers alike is to make toothpaste that clean well and at the same time; virtually non-abrasive to the dental hard tissues (Lippert 2013). Recently, the use of eggshell powder has attracted much interest due to its relatively low abrasivity. Onwubu *et al.* (2017b) demonstrated that a dental abrasive material made from eggshells was effective in the polishing of dental acrylics. Chen (2008), on the other hand, revealed that eggshells of particle size between 0.1 μm to 10 nm produced an ultra-fine powder, which made them useful in toothpaste and cosmetics.

The above studies on the abrasive characteristics of eggshells particularly in dental applications, strongly suggest that developing a desensitising toothpaste from eggshell powder and titanium dioxide may have less harmful effects on the enamel and exposed dentin. This is also in line with the report by Moore and Addy (2005) who observed that calcium carbonate containing toothpaste have low abrasivity on the enamel. Although clinical evaluation of all the commercially available toothpaste is not feasible, Hara and Turssi (2017) have suggested that laboratory tests provide a good indication of toothpaste abrasive potential in relation to enamel and dentin. The authors' suggestion is highly pertinent in this study particularly in evaluating the abrasive effect of EB@TiO_2 paste on enamel.

2.7 Method of validating EB@TiO_2 Remineralisation and acid resistant Characteristics

As earlier stated in this Chapter, tooth demineralisation and remineralisation is a constant dynamic process occurring in the oral cavity (Neel *et al.* 2016). This dynamism is being promoted by the amount of available calcium and phosphate as well as the pH of the oral environment (Cury and Tenuta 2009; Li *et al.* 2014). Consequently, and in line with the advice of Neel *et al.* (2016), understanding the process of mineral deposition is paramount for validating the fitness of purpose of EB@TiO_2 desensitising toothpaste in the treatment DH as well as the prevention of tooth demineralisation. Trullols (2006) claimed that the process of measuring the effectiveness of any material relates closely to the concept of 'fitness of purpose'. As highlighted in the Orange book of the International Union of Pure and Applied Chemistry (Inczédy, Lendyel and Ure 1998), the "fitness of purpose" of a material is the degree to which data produced by measuring process enables a user to make technically and administratively correct decisions for a defined purpose. In reviewing the literature on methods and techniques used to study dental hard tissues, the following techniques namely: Scanning Electron Microscopy; Atomic Force Microscopy; Profilometry; and Raman Spectroscopy opens a new angle for the understanding of tooth mineralisation (Gronwald *et al.* 2018). The stated techniques will provide confirmation that EB@TiO_2 meets its intended use (International Standard Organisation 2005).

2.7.1 Scanning Electron Microscope

In terms of the study of DH, Kulal *et al.* (2016) moot that techniques such as hydraulic conductance test and scanning electron microscope (SEM) have been employed to either measure fluid movement before and after or the occluding characteristic of desensitising agents on dentine. Unlike the SEM, the hydraulic conductance has the disadvantage in that the dentin samples cannot be re-examined (Ahmed *et al.* 2005). As a consequence, studies on the effectiveness of desensitising agents have been limited to SEM observation of the dentin surface (Gholami *et al.* 2011; Kulal *et al.* 2016).

Importantly, SEM techniques (Figure 2-9) can provide accurate information about the surface topography as well as the composition of dental hard tissues by focusing the beam of electrons at the specimen surface (Field 2012). Equally important, SEM images have a large depth of field which can give a high resolution in 2-D images (Rodriguez-Vilchis *et al.* 2011). Added to this, conventional SEM required a certain degree of sample preparation, hence, it is recommended that the surface of the specimen be coated with a material that is electrically conductive to prevent the accumulation of electrostatic charge and operated in a vacuum environment (Sharma *et al.* 2010). This material is usually gold or carbon, and the specimens will undoubtedly be irreversibly altered during the desiccation and sputtering process (Sharma *et al.* 2010). Gold–sputtering the specimen is pertinent to this study, particularly when microscopically observing the degree of remineralisation and or demineralisation of the dentin and enamel surfaces.

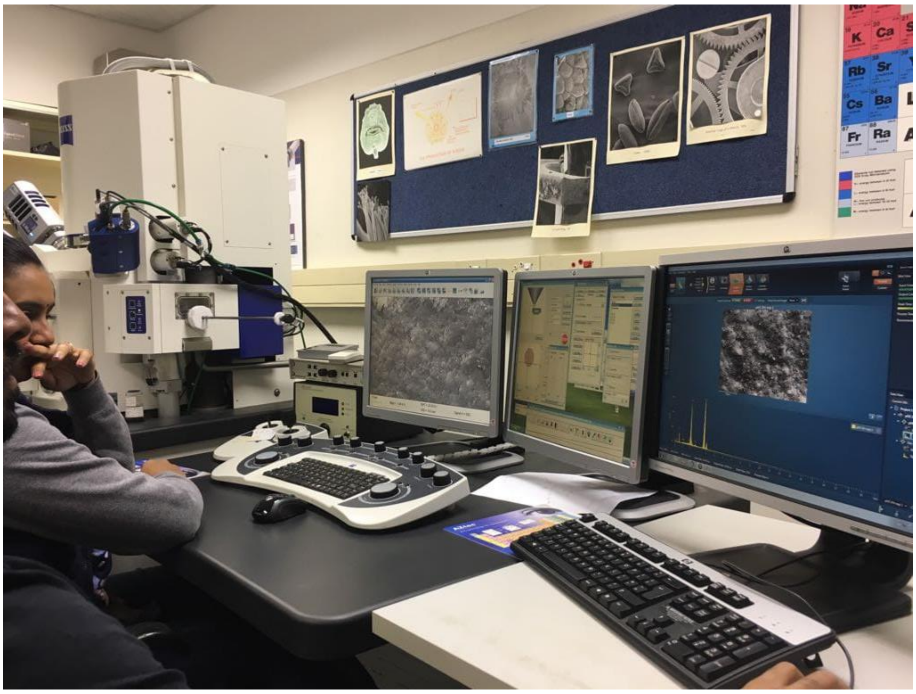

Figure 2- 9: SEM observation of samples

2.7.2 Atomic Force Microscope

The Atomic Force Microscope (AFM) is another useful device that has generated increasing attention for the study of dental hard tissues (Watari 2005; Pan *et al.* 2008; Gronwald *et al.* 2018). Figure 2-10 depicts the principle of AFM setup. Gautier *et al.*

(2015) espoused that an AFM consists of a piezocontroller on which a cantilever is typically attached (Figure 2-10A). Apart from these, there is a laser that reflects off the cantilever surface, a four-quadrant, and a mirror which function is to change the angle of the laser beam and reflects same onto the photodiode (Gautier *et al.* 2015). Gautier and his co-workers had observed that the reflection of the laser beam onto the photodiode results in subnanometer spatial and millisecond temporal resolution (Gautier *et al.* 2015). Ideally, and as shown in Figure 3-10B, the AFM is placed on the x-y stage, which holds the sample. Gautier *et al.* (2015) revealed that the stage is attached to an inverted microscope and installed on a vibration isolation table to avoid possible interferences.

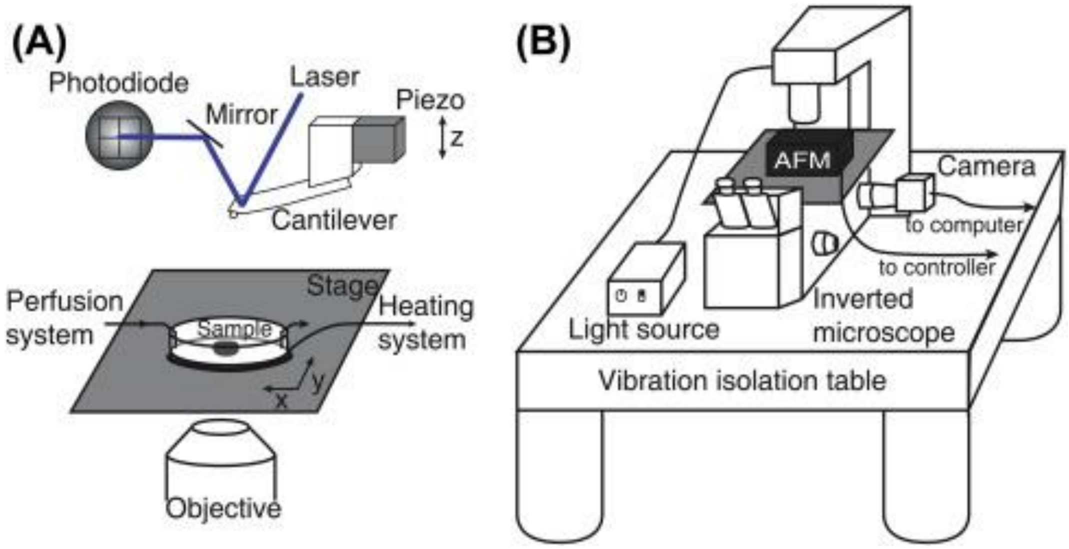

Figure 2- 10: Principle and experimental setup of AFM measurement on biological samples showing (A) Schematic drawing of an AFM setup; (B) overview of the experimental setup (Source Gautier et al. (2015))

Owing to the above-described setup, AFM has been widely applied to simultaneously obtain qualitative (3D image) and quantitative analysis of dental tissues at the nanoscale, thus providing an important understanding of oral disease treatments and progression as well as biomedical device coating (Bozec and Horton 2006; Gaikwad and Sokolov 2008). Rodriguez-Vilchis *et al.* (2011) argued that AFM is an important tool for studying biological tissues and materials due to its higher resolution of 3D image when compared against the 2D image typically observed with SEM. More so, AFM analysis requires minimal sample modification and alteration (Sharma *et al.*

2010). Accordingly, it is proposed that the combination of both SEM and AFM may improve the observation of morphological changes of the enamel and dentin surfaces (Rodriguez-Vilchis *et al.* 2011). This, and as noted by Rodrı´guez-Vilchis *et al.* (2011) could result in a more synergistic achievement by taken cognisance of the advantages inherent in each technique.

2.7.3 Profilometry

With reference to the assessment of the abrasive actions of EB@TiO_2 on tooth enamel, it worth stating that relative dentin abrasivity (RDA) and or relative enamel abrasivity (REA) are the standard test recommended by the International Organization for Standardization (2017) (ISO 11609). Although the RDA or REA are considered a useful tool for determining the abrasiveness of desensitising paste (González-Cabezas 2010), the technique, however, involves a radiation-based method and thus requiring regulatory clearance for isotope use (Hara and Turssi 2017). As a consequence, the ISO 11609 suggested alternative techniques for assessing the abrasiveness of toothpaste by means of profilometry analysis. As reported by Hara and Turssi (2017), the profilometry has the advantage over RDA techniques since it does not require irradiated specimens. More so, previous studies (Sabrah *et al.* 2013; Schneiderman *et al.* 2015) that compared the effectiveness of RDA and profilometry find no differences between the two methods.

In the recent decade, literature has documented the widespread use of the profilometer in assessing the abrasivity of desensitising toothpaste (Johannsen *et al.* 2013; Arnold *et al.* 2016; Athawale, Srinath and Chowdary 2018) thereby supporting its use in this study. Field (2012: 25) explained that profilometer can measure the surface changes of dental tissues directly or indirectly using a contacting stylus and a laser profilometer, respectively. It is worth noting that the current ISO 5436-1 (International Standard Organisation 2001) advised using the stylus profilometer. Srividya, Chandrasekharan and Jayakar (2011) hinted that stylus profilometry measurement involves traversing the surface with a diamond-tipped stylus, usually at a fixed radius 1.5-2.5µm. As highlighted by the ISO 3274 (International Standards Organisation 1975) standard manual, the chisel-point tips (0.2µm x 2.5µm) is mainly used for detecting raised areas on a surface, whereas the conical tips are almost exclusively used for micro-roughness measurement. Stachowiak, Batchelor and Stachowiak (2004) also reported that the loading weight on the stylus ranges from 0.05-100mg. The abrasivity of EB@TiO_2 on the prepared enamel specimens in this study will be measured using a contact stylus profilometer. Field (2012) revealed that during use, the vertical motion is dragged across the surface and is transformed into an analogue/digital signal. This will be taken into consideration when assessing the abrasiveness of EB@ TiO_2 with a profilometer.

2.8 Predictive model for dentin remineralisation

Importantly, mathematical modelling offers a different research perspective by overcoming some of the problems frequently encountered in an experimental study (Ilie, van Loosdrecht and Picioreanu 2012). Essentially, using numeral tools, Ilie, van Loosdrecht and Picioreanu (2012) assumed that it is possible to fashion out a controlled environment to address the challenges of long time period needed to effectively study the biological process. This process, and as advice by Ilie (2014) must be based on experimental studies. The author emphasised that the input parameters under investigation (for example solute, microbial, transport, etc.) should be determined experimentally, as this would ultimately impact on quality of the model. This study will be taken cognisance of the input parameters such as the dentin tubules radius in order to help predict the duration of remineralisation of dentin tubules. This is in accordance with the report by Davari, Ataei and Assarzadeh (2013) that the rate of dentinal fluid flow depends on the fourth power of tubule's radius.

In the last decade, different mathematical model has been proposed by various scholars to investigate dental hard tissues, most nevertheless, focus largely on dental caries and tooth demineralisation process (Ilie, van Loosdrecht and Picioreanu 2012; Fábregas 2014; Ilie 2014). Despite the numerous model developed to study dental tissues, there is limited evidence that suggests the use of a mathematical model to predict the remineralisation potentials of desensitising agents on dentin tubules. This study therefore proposed logistic equation as a tool for the prediction of the effectiveness of desensitising agents in occluding dentin tubules.

In addition, and in view of developing a predictive model for dentin remineralisation, the following input parameters are critical for the mathematical equation:

1. **Size of the dentine tubules (S)**: Davari, Ataei and Assarzadeh (2013) suggested that tubules in exfoliated teeth (sensitive tooth) are eight times more numerous, twice larger in diameter and are open, whereas tubules in a non-sensitive tooth are less numerous, smaller, and usually blocked.
2. **Amount of calcium and phosphate deposits (A):** Shetty and Kundabala (2013) explained that dentine remineralisation is the amount of calcium and phosphate ions being deposited into crystal voids, for example, in exposed tubules.
3. **Time (t):** Here time will be an important parameter to predict the duration of remineralisation.

Although models have great prospect to improve development and implementation of treatment, Murphy, Jaafari and Dobrovolny (2016) warned that this can only hold true when models provide accurate predictions. The next section will examine the predictive ability of the logistic equation model.

2.8.1 Logistic equation

The logistic equation was first proposed by the seminal work of Pierre-Francois Verhulst (1844-1845). Verhulst derived the logistic equation to describe the self-limiting growth of biological population (Kyurkchiev and Markov 2016). Interestingly, Sweilam, Khader and Mahdy (2012) assert that the logistic equation is described by first-order ordinary differential equation. Their report resonates further with Murphy, Jaafari and Dobrovolny (2016) who noted that the logistic equation is formalised by the differential equation and it is typically represented as follows:

$$V - aV\ (1 - V/b \qquad (1)$$

Accordingly, Murphy, Jaafari and Dobrovolny (2016) observed that the logistic model describes the growth of a population that is limited by a carrying capacity of *b*. According to their report, the logistic equation assumes that the growth rate decreases linearly with the size until it equals zero at the carrying capacity.

Ever since the discovery, the logistic equation has been extensively used in many scientific fields such as ecology; chemistry; population dynamism; mathematical psychology; political science; geoscience; statistics; economics and sociology etc. (Alt and Markov 2012; Bersani and Dell'Acqua 2012; Markov 2014; Radchenkova *et al.* 2014). In ecology, for example, logistic equation has been widely used to model the population growth where the rate of reproduction is proportional to both the existing population and the number of resources available (Sweilam, Khader and Mahdy 2012). This is expressed mathematically as follows:

$$dp/dt = rP.(1 - \frac{P}{k(t)}) \quad (2)$$

Where P represents population size, r is the constant that defines the growth rate, K is the carrying capacity, and t represents the time

Another typical application of the Logistic equation is in medicine, where the Logistic differential equation is used to model the growth of tumors or to study the reaction of pharmacokinetic (Kyurkchiev and Markov 2016). Here, the application of the logistic equation can be considered an extension of the above mentioned use in the framework of ecology where d(t) the size of the tumor at time t (Sweilam, Khader and Mahdy 2012). Given the predictive power of the logistic equation, this study uses the logistic equation to study the remineralisation capabilities of three desensitising paste namely EB@TiO_2, Pro-argin, and bioactive glass (NovaMin) in saliva and without saliva.

In summary, this chapter foreground the different approaches that have been used for the management of DH. The foregoing chapter also outlined the characteristics of eggshells in terms of structure, and mineral content. Equally, the antibacterial and acidic resistant properties of titanium dioxide were presented. These properties were noted as being fundamental in the development of a new desensitising paste using eggshell and titanium dioxide (EB@TiO_2). The foregoing reviewed literature further highlighted an eco-friendly strategy for the preparation of EB@TiO_2 through the mechanochemical method. Furthermore, instruments for validating remineralisation of dental tissues were reviewed as it is an underpinning concept with regards to assessing the effectiveness of EB@TiO_2 for the management of DH. Overall, the predictive power of the logistic equation was explained, as it is a tool for predicting the remineralisation potential of EB@TiO_2. The next chapter will provide the underlining theoretical framework adopted to evaluate the product quality of EB@TiO_2.

Chapter Three –Dental public health and theoretical considerations for the study

One of the pivotal goals of the research is to contribute to the existing body of knowledge and identify its public health value. This chapter examines the value of recognising dentine hypersensitivity as a public health concern. It is equally important to identify the theoretical basis that underpins the research process.

3. 1 Relevance to dental public health

According to Naidoo (2015), the pattern of oral diseases globally continues to echo extensive inequality in access to public preventive and dental care, and DH is no exception. Dentine hypersensitivity [DH] is a common occurrence and notable painful conditions among dental patients (de Melo Alencar *et al.* 2019). Saeki *et al.* (2016) documents that more than 80% of children and up to 43% of adult's population suffers from dental pain associated with DH. More so, Miglani, Aggarwal and Ahuja (2010) noted that DH can affect dental patient of any age. From epidemiology and etiological perspective, Miglani, Aggarwal and Ahuja (2010) postulate most affected patients are in the age group of 20-50 years, with a peak between 30 and 40 years of age. It is, therefore, reasonable to assume that the categories of affected patients fit into the working force of any society, which could in turn - negatively impact on the individual lifestyle and productivity if left untreated (Hirsiger *et al.* 2019).

Moreover, it has been argued in the literature that the global distribution of oral diseases and the severity of their consequences constitute a pandemic condition (Edelstein 2006; Dye 2017). Despite the said concerns, the treatment of oral diseases still remain expensive and may be particularly inadequate in low and middle-income countries in underserved populations (Petersen 2005; Naidoo 2015). More worrisome is that inequalities in oral health care continue to widen amongst the disadvantaged (children and women) and vulnerable groups (elderly) who still experience the highest burden of oral diseases (Petersen *et al.* 2005; Singh, Myburgh and Lalloo 2010; Jin *et al.* 2011).

Against the above backdrop, this study is designed to use waste eggshell in the development of desensitising agents for the management of DH. More especially, the need to address the social economic inequality in the access of oral health care is underpinning motive behind the development of a new desensitising paste using eggshell powder and titanium dioxide (EB@TiO_2). This strongly aligns with the Alma Ata Declaration of 1978 that emphasises the concept for "Health for All" (Hall and Taylor 2003).

3.2 Theoretical basis to guide this study

This study embeds the social justice principle as the underlying core to address the dire gap in the accessibility and affordability of oral health care (Naidoo 2015), through the development of a novel product to address DH. This study is also premised on the principles of the hydrodynamic theory postulated by Brännström, Lindén and Åström (1967) as the underpinning assumption to assess the quality of EB@TiO_2 to occlude dentin tubules.

3.2.1 Social Justice

Van den Bos (2003) defined social justice as "*the fair and equitable distribution of power, resources, and obligations in society to all people irrespective of their gender*, religious affiliation, age, sexual orientation, race or ethnicity, and ability status". The salient point emerging from Van den Bos interpretation of social justice underscores the importance of inclusion, collaboration, and more especially-equal access and opportunity. This same tenet guides the public health system that strongly emphasises on the doctrine of egalitarian that all human persons are equal in fundamental worth or moral status and, hence, there health needs including their oral health should be met (Naidoo 2015).

3.2.2 Social justice and oral health inequality

In South Africa, the legacy of the apartheid created inequality in oral health as different separate societies are exposed to unequal health and other resources (Myburgh *et al.* 2005). Although there have been conscientious efforts by the South Africa government to reverse some of the adverse effects of the apartheid laws through the provision of access to basic health care, this progress, is, however, undermined by the challenge of high inequality, high poverty and high unemployment (Sulla and Zikhali 2018). More concerning is that that there is still general neglect of oral health in South Africa (Ramphoma 2016). This has contributed to the lack of oral health facilities and workforce, and worse by the unequal distribution of dental services in the country (Ramphoma 2016).

3.2.3 Addressing inequality in oral health

Access to oral health should not be limited to dental treatment and should also be expressed in the access to preventive measure against oral diseases (Naidoo 2015). Resonating with Naidoo, Ramphoma (2016) advocated for an alternative measures to access oral health care that will focus on prevention of oral disease and oral health promotion, as opposed to the existing curative approach in the management of oral health.

From an economic point of view, curative treatment of oral diseases is economically draining for the country, with some estimates suggesting that the total costs for providing curative dental care would overshadow the whole healthcare budget of low-income countries (Kathmandu 2002). Equally concerning, Thorpe (2006) points out that curative treatment is technically challenging and requires the use of expensive equipment and highly skilled professionals. This is a response to call made by Schmidlin and Sahrmann (2013) that a noninvasive, nonhazardous, reversible, easy to perform, and inexpensive material is the most desirable option for the management of DH.

The next section examines the hydrodynamic theory on which the validity of EB@TiO_2 is benchmarked (Figure 3-1).

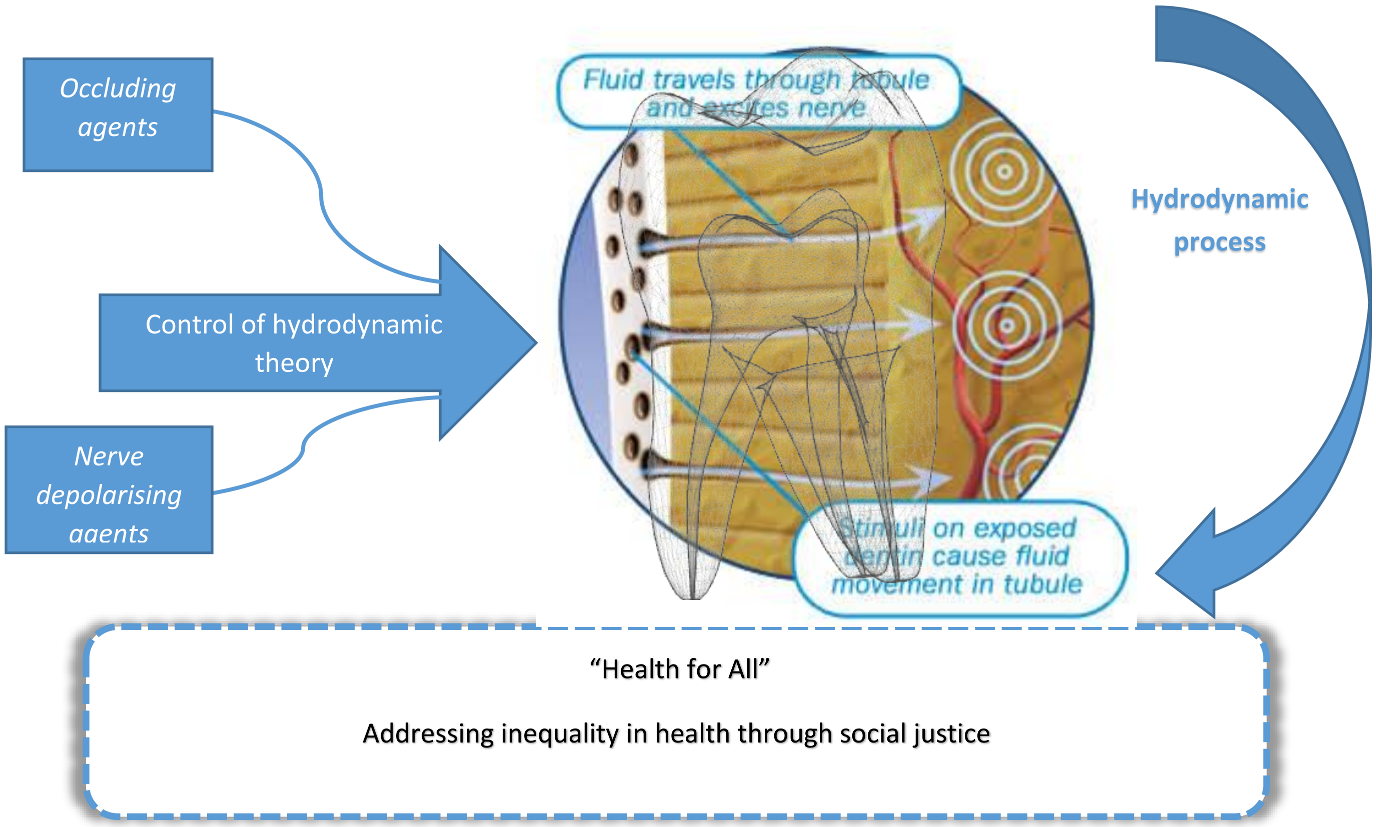

Figure 3- 1: Showing the underlining concept of developing EB@TiO_2 to address the hydrodynamic principle is anchored on the social justice principle (Adapted by the researcher)

3.3 Hydrodynamic theory

As reported by Addy (2002), Cummins (2009) and Schiff *et al.* (2009), the hydrodynamic theory proposed by Brännström, is widely regarded as the accepted theory for DH. The underlining assumption is that an external stimulus such as temperature, physical or osmotic changes triggers a pressure change in the dentin fluid. As a consequence, fluid movement transmits a signal to the odontoblastic process, which carries the stimulus from the tooth surface toward the afferent nerve ending in the dentin tubule thereby triggering pain (Brännström 1986).

According to Panagakos, Schiff and Guignon (2009: 3), the pain caused by the movement of fluid in the dentine tubule is transient that is once the stimulus is removed or dissipates the pressure within the tubule returns to normal and the pain subsides. Schiff *et al.* (2009) noted that the management of DH typically aims to control the hydrodynamic mechanisms. Hence, and as indicated by Panagakos, Schiff and Guignon (2009), and Schiff *et al.* (2009) approaches to control the hydrodynamic mechanisms has resulted in the development of two classes of products namely: (1)

products or agents that reduce fluid flow within the dentine tubules, which in turn blocks or occlude the external stimuli, and (2) agents or products that interfere with the transmission of nerve impulses. It is envisaged that the nano-sized eggshell and titanium dioxide desensitising (EB@TiO_2) toothpaste will effectively treat DH by remineralising damaged teeth.

In summary, and despite the policy and advocacy for Health for All, oral health care particularly in the developing and low-income countries like South Africa continues to reflect inequalities in respect to resources distribution. In an attempt to address this gap, a low cost effective material is desirable. Hence, the hydrodynamic theory guides the research in terms of product validation. The next chapter describes the research design and methodology adopted in the present study. In particular, the development and testing of the EB@TiO_2 will be discussed in detail.

Chapter Four – Research Methodology and Design

The research methodology and design adopted in this study is detailed in this chapter. The phase one of the study conducted will first be described, particularly highlighting the preparation and the modification of eggshell-titanium dioxide composite (EB@TiO_2). Subsequently, the methodology used in phase two and three, that is the various experimental work and analyses that were conducted, will be described in-depth.

4.1 Introduction and Background to the research methodology

Research is generally stimulated by a number of methods. Studies by Welman, Kruger and Mitchell (2005), and (Berg and Latin 2008) noted that these methods are the scientific method of inquiry and critical thinking, deductive reasoning, inductive reasoning amongst others. Deductive reasoning provided the basis for the initiation of this study. Nakashima *et al.* (2009), and Rahardjo *et al.* (2015) argued that using occluding products that physically block the exposed dentine tubules is highly effective in remineralising enamel lesions, particularly in toothpaste. Cutler (2014) further posited that nano-sized titanium dioxide, when used together with abrasive agents such as calcium carbonate, has the potential to occlude open dentinal tubules, which can effectively reduce DH.

Moreover, and heeding the advice by Schmidlin and Sahrmann (2013) that an inexpensive, nonhazardous, and noninvasive material should be developed for the management DH, this study sought to develop a new desensitising agent using waste eggshells and titanium dioxide (EB@TiO_2). This study, therefore, follows a quantitative research approach and an experimental research design strategy. Johnson and Christensen (2008) alleged that a quantitative approach tests "hypotheses with empirical data to see if they are supported". The authors also alluded that an experimental research design enables the researcher to manipulate the independent variable and measure the dependent variable in order to "identify cause-and–effect relationships" (Johnson and Christensen: 2012: 33). To facilitate the proposed research design and methodology, a three-phase research methodology was considered most appropriate (Figure 4-1).

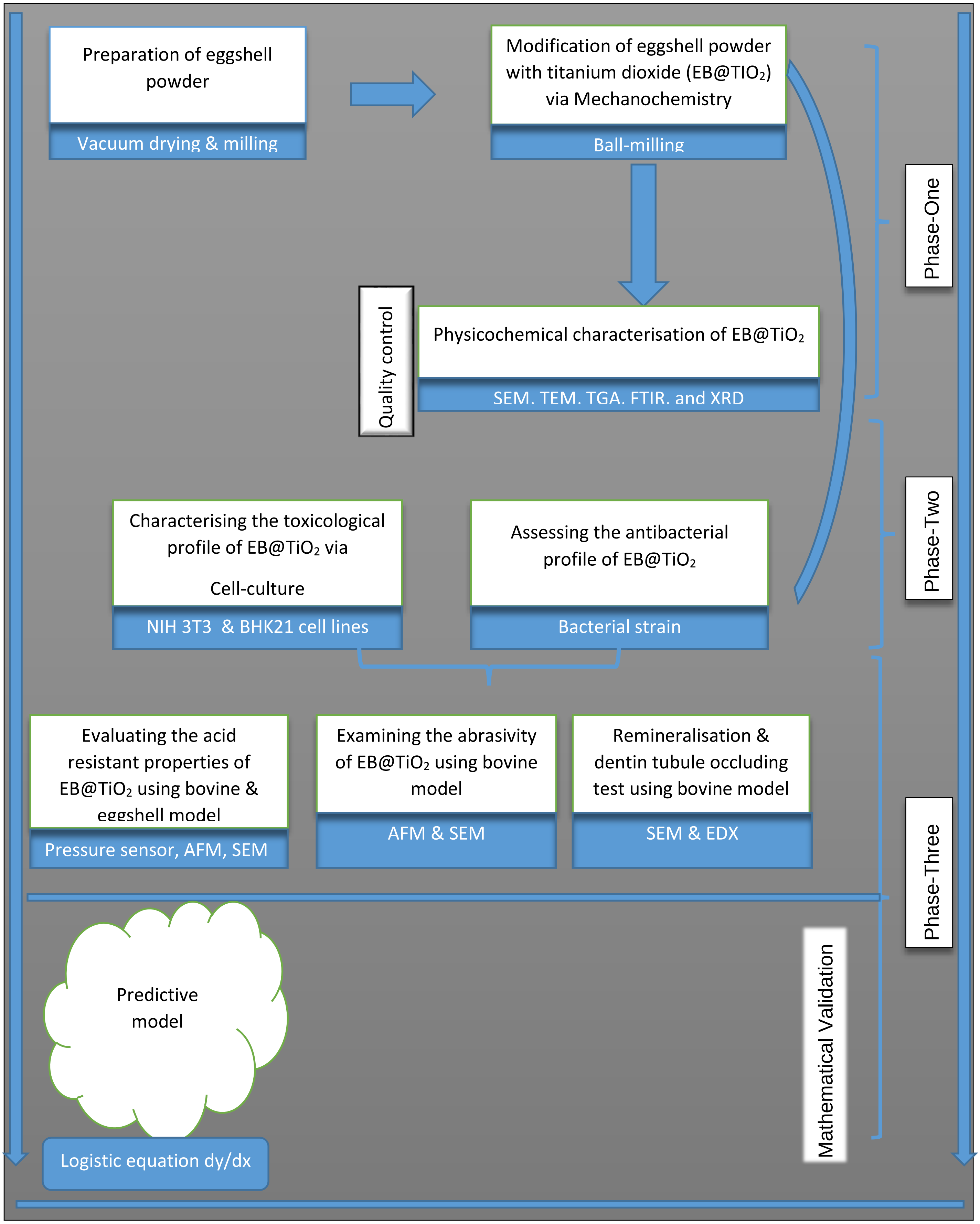

Figure 4- 1: Experimental research design showing the interrelationship in the research study phase.

4.1.1 Delimitation

Regardless of the type of avian eggshell, several studies (Stadelman 2000; Hincke *et al.* 2012; Patrick, Aigbodion and Hassan 2012) have shown that eggshell is a natural bioceramic composite with a unique chemical composition of high inorganic (~ 95% of calcium carbonates in the form of calcite) and ~ 5% of organic (type X collagen, sulphated polysaccharides) components. It must be noted that chicken eggshell was used as a source of calcium carbonate in developing EB@TiO_2 desensitising agent. The abundant availability, and low cost of chicken eggshell in South Africa deemed it appropriate to be used in this study.

4.2 Phase One: Development and Characterisation of a modified Nano-sized EB@TiO_2

In accordance with the South African National Standards (SANS 1302: 2008), and the International Standards Organisation (ISO 11609:2010) ingredients used in the production of desensitising paste, EB@TiO_2 was developed and characterised as described in the sections below.

4.2.1 Preparation of eggshell powder

In preparation of eggshell powder, eggshells were first collected from food outlets within Durban, South Africa. All preparation and ball milling of the eggshell powder was done at the Environmental Research Group Laboratory (Durban University of Technology, Durban, South Africa).

As proposed by Onwubu (2016) the eggshell powder was prepared based on the formulae below:

- **Washing and disinfecting of the eggshells:** Eggshells were disinfected by storing the eggshells in a diluted solution of household sodium hypochlorite for six hours. This was to help remove any form of contamination from the source of collection.

- **Vacuum Drying:** To burn out the membranes the eggshells were vacuum dried for ± 6-9 minutes at 250°C. These were then allowed to cool before milling.

- **Ball Milling:** The vacuum dried eggshells were subsequently ball-milled by placing 30g of the eggshell in a 250ml stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10mm diameter and dry-milled in a planetary ball mill (Retsch ® PM 100) at 400 rpm for 20 minutes.

- **Mechanical Sieving:** As depicted in Figure 4-2, and following the American Society for Testing and Materials (ASTM C136-06) standard for sieving abrasive powders, the milled eggshell powder was sieved to a particle size of ≤ 25μm using a mechanical sieving shaker (Retsch AS 200, Germany).

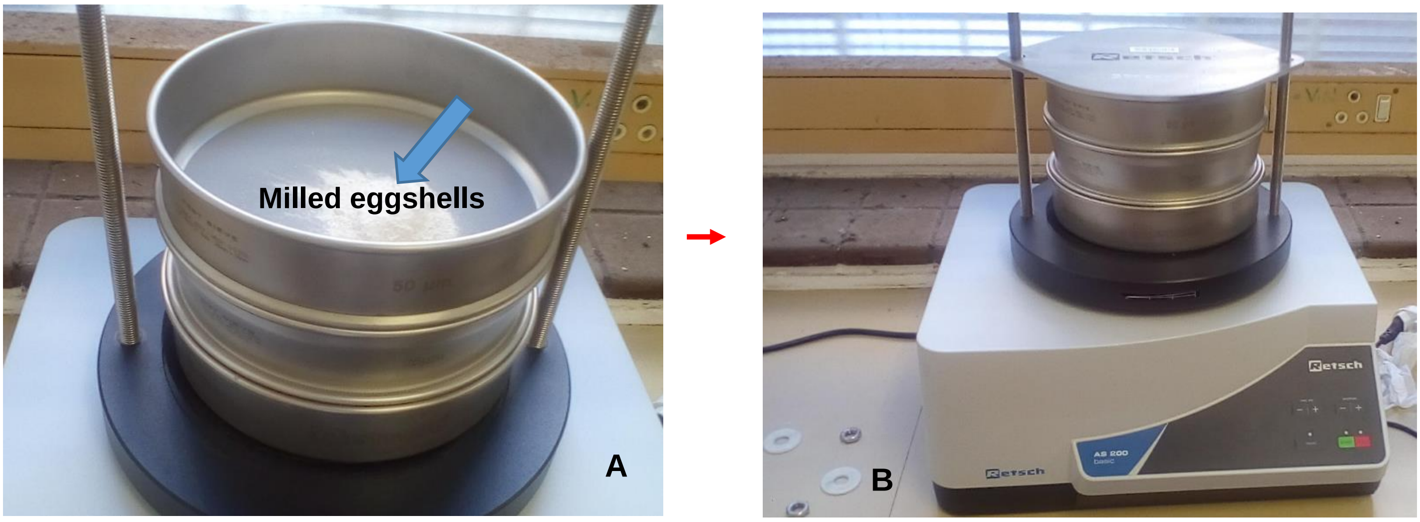

Figure 4- 2**:** Mechanical sieving process: **(A)** Milled eggshells; **(B)** Shaker

4.2.2 Preparation and Modification of Nano-sized Dental Eggshell-Titanium dioxide composite (EB@TiO$_2$)

The prepared eggshell powder obtained above was further modified with Food grade anatase titanium dioxide (CAS No: 13463677; Sigma-Aldrich Germany). The eggshell powder (≤ 25μm) and titanium dioxide (≤15μm) mixing ratio was optimized following the procedure reported by Lin, Dong and Jiang (2009). 20g of the fine eggshell powder was modified by adding 5g of food grade anatase titanium dioxide. The mixture was subsequently ball-milled for 200minutes to obtain a nano-sized eggshell-titanium dioxide material.

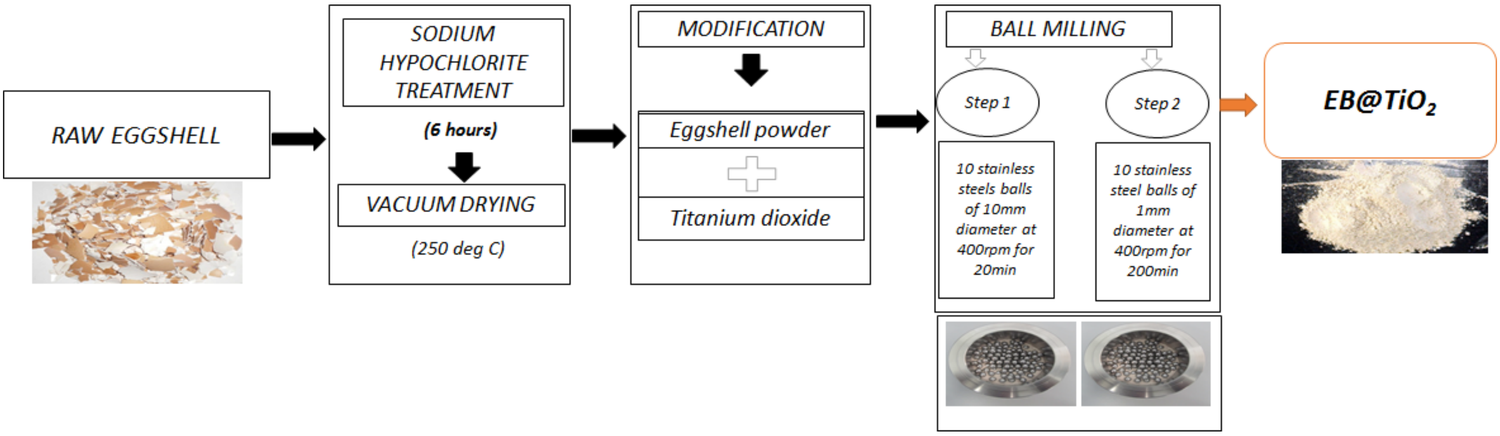

Figure 4- 3: Schematic Illustration of the preparation and modification of EB@TiO_2

4.2.3 Physicochemical characterisation of EB@TiO_2

In complies with the international standards for quality management and quality assurance (ISO 9001: 2008), the particle size, shape, phase change, and crystallinity of EB@TiO_2 desensitising agent were determined by using the various analytical techniques. These are detailed in the sections below.

4.2.3.1. Fourier Transform Infrared Spectroscopy Analysis

The infrared spectra were measured using a Perkin Elmer Universal ATR spectrometer to identify the functional group constituents of eggshell powder, titanium dioxide, and EB@TiO_2. The ATR was carried out to confirm the modification of the eggshell powder with titanium dioxide. A very small amount of each sample was placed in the sample holder. An initial background check was performed before scanning at a range of 400-4500 cm^{-1}, and at a resolution of 4 cm^{-1}.

4.2.3.2. X-Ray Diffraction Analysis

The early work of Dutrow and Clark (2008) alleged that XRD is the most commonly used method for identification of unknown crystalline materials and their mineralogical composition. In line with these, X-ray diffraction (XRD) analysis was performed to observe the possible changes in crystallinity between the eggshell powder, titanium dioxide, and EB@TiO_2. The XRD patterns were recorded using a diffractometer (PANalytical-Empyrean instrument; Co radiation 1.54056 Å) and analysed between 0-90^o (2 theta). The voltage, current and pass time used were 40 Kv, 40mA, and 1s, respectively.

4.2.3.3 Energy Dispersive X-Ray Spectroscopy and Scanning Electron Microscope Analysis

Energy dispersive x-ray spectroscopy was used in conjunction with scanning electron microscope (Field Emission-Carl Zeiss) operating at controlled atmospheric conditions at 20 kV to examine the surface morphology and the elemental composition of the eggshell powder, titanium dioxide, and EB@TiO_2. These were done to observe microscopically, the modification of eggshell powder with titanium dioxide. As a proxy measurement, pre and post agitation of EB@TiO_2 after 30 minutes with ultrasound technology (UP4DOS, Hielscher) was carried out to establish the strength of modification (Figure 4-4). As advised in the literature (Sharma *et al.* 2010), the surface of the samples was coated with a thin, electric conductive gold film to prevent the build-up of electrostatic charge before the SEM observation.

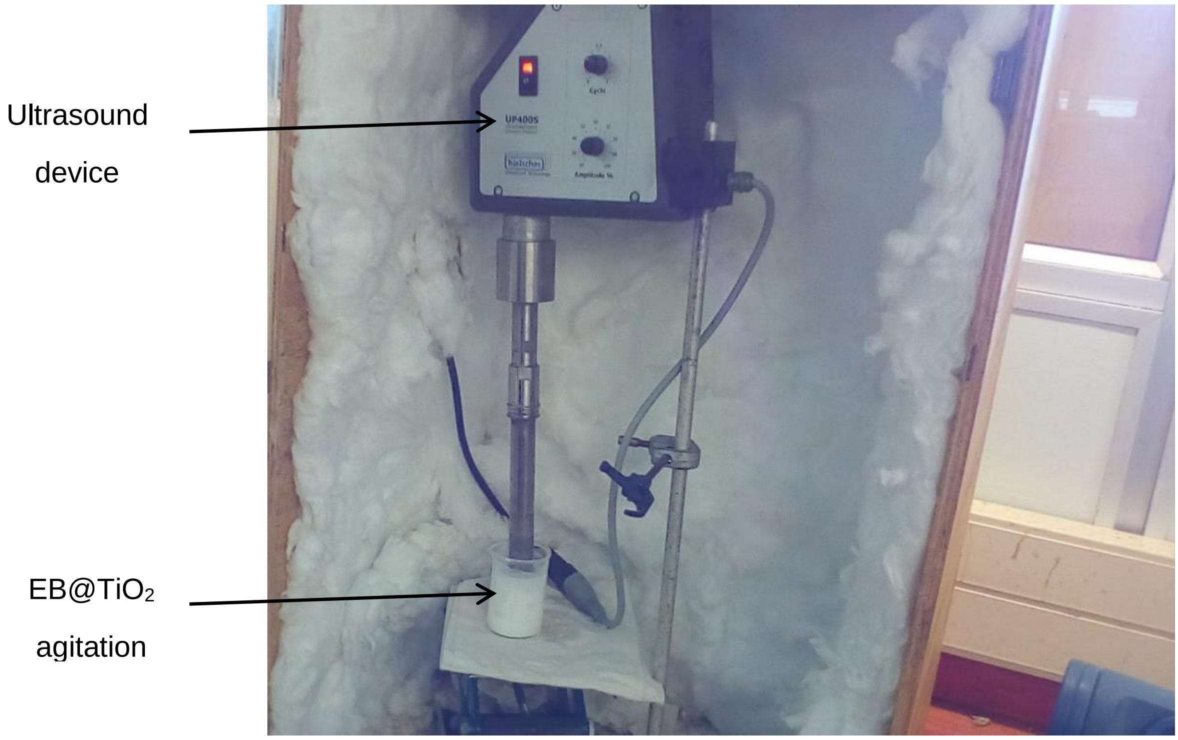

Figure 4- 4: Agitation of EB@TiO_2 using ultrasound technology

4.2.3.4. High Resolution Transmission Electron Microscopic Analysis

A High Transmission Electron Microscope (HRTEM) was used to observe the particle size, shape, and distribution of EB@TiO_2. Very small quantities of EB@TiO_2 were dispersed in 10ml ethanol and sonicated at 10kv for 10 min. Subsequently, thin cross-sections of cryo-microtomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids with a mesh size of 100x400 dimensions. The analysis was conducted using a high transmission electron microscope (HRTEM-Philips CM 120 model) at 120 kV. The use of HRTEM to observe the morphological characteristics of EB@TiO_2 in this study was in align with the report by Howe, Fultz and Miao (2012) that TEM is the foremost instrument for understanding the internal microstructure of materials at the nanometer level.

4.2.3.5. Computation of TiO_2-EB Structural Interaction using Material Studio and EDX Mapping

Material studio software (version 6) was used to analyze the structural interaction of the calcite and titanium dioxide component of EB@TiO_2. EDX mapping was further used to validate the computation interaction.

4.2.3.6 Thermo-gravimetric Analysis

The early report by Sauerbrunn and Gill (1994) moots that thermal degradation of a material is an indication of the material kinetic decomposition, and thus can be highly useful to make a prediction of the shelf life of the material. In light of these, thermal degradation and stability of the EB@TiO_2 was studied using a Thermo-gravimetric analysis (TGA). The thermal stability was measured using a TA instrument (Thermal Universal Analyser V4.5A). The test was performed under a dry nitrogen gas flow at the rate of 100mL/min from 20^oC at a heating run of 10^oC/min.

4.3 Phase Two: Toxicological and Antibacterial Assessment of EB@TiO_2

This phase ensures that the EB@TiO_2 desensitising agent is compliant with ISO/TR 1321 in terms of the potential risks and safety concern of the use of nanomaterials. The microbial assessment of the EB@TiO_2 was done at the Biotechnology Laboratory (Durban University of Technology, Durban, South Africa). The Advanced material Division (MINTEK) Randburg, South Africa assisted in the cytotoxicity assessment of the EB@TiO_2.

4.3.1 Cytotoxicity assay

Eggshell powder, titanium dioxide, and EB@TiO_2 samples were dispersed in Dimethyl Sulfoxide (DMSO), before the administration to the cell line. An MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used to evaluate the change in cell viability. Auranofin was used as a negative control. The samples were tested across 2 plates in duplicate (n=6) and the average value reported. NIH 3T3 (mouse fibroblast) and BHK 21 (hamster kidney fibroblast) cells were grown using normal tissue culture techniques. The cells (1 x 105 cells/ml) were incubated in 96 well plates at 37°C overnight, with the subsequent addition of the supplied compounds, in concentrations of (100 µg/ml, 50.0 µg/ml, 25.0 µg/ml, 12.5 µg/ml, 6.25 µg/ml, and 3.13 µg/ml). The cells were left to incubate for 4 days. Thereafter, MTS (5 µl) was added to the cells. The absorbance values were measured at 490 nm after 1h, 2h and 4 hour incubation periods, averaged and the viability curves drawn up.

4.3.2 Antimicrobial Assessment

The inhibitory properties of EB@TiO_2 against both Gram-positive and negative bacterial strain were studied the using disk diffusion method. The Bacterial strain was obtained from Anatech supplies, South Africa. Thereafter, pure colonies from freshly grown strain *E.coli* (ATC 25922), and *B. Cereus* (ATC 10876) were isolated and subsequently transferred from the plates into a sterile normal saline solution and vortexed to form bacterial homogenous suspensions. The turbidity was then adjusted to 0.5 McFarland standard units, and the suspensions were poured over Mueller–Hinton agar (MHA) plates. Sterile filter paper disks with a diameter of 6 mm were placed over these plates. The sterile disks were impregnated with 20 µL of the tested compounds (10 mg/mL dissolved in DMSO). Positive control (Ciprofloxacin) and negative control (sterile distilled water) were used. The impregnated plates were incubated at 37°C for 24 hours. in line with the procedure described by Wiegand, Hilpert and Hancock (2008), the inhibition zones were calculated and the value estimated in millimeter.

4.4 Phase Three: Assessing the "fitness of purpose" of the EB@TiO_2

This phase assesses the fitness of purpose of the prepared EB@TiO_2. The phase examines the protective effect of EB@TiO_2 against erosive challenge (Paper I, II, III, and IV), its abrasiveness on bovine enamel (Manuscript III), the dentin tubule occlusion characteristics (Paper V. VI, and Manuscript I), and the logistic equation model that predicts the remineralisation of desensitising paste (Manuscript II).

4.4.1 Evaluation of Acid Resistant Properties for EB@TiO_2

The acid resistant characteristics of EB@TiO_2 were evaluated by comparison using simulated eggshell enamel model and bovine enamel, respectively. It was assumed that if EB@TiO_2 has improved acid resistant properties then it would be a suitable material for the remineralisation and repair of damaged teeth.

4.4.1.1 Evaluation the acid resistant properties of EB@TiO_2 using Eggshell model

Eggshell enamel samples were simulated *in vitro* using EB@TiO_2 and eggshell powder (control). 3 g of each brand of toothpaste (See Paper I, II, and III) were dissolved in a beaker containing 100 cm^3 deionized water by constant agitation using magnetic stirrer at 800 rpm for 20 minutes. 1 g of the prepared eggshell powders and EB@TiO_2 was added to the beaker containing the dissolved toothpaste, respectively. These were further agitated for 8 hours at a speed of 800 rpm. The mixtures were then filtered and subsequently oven dry at 60 degrees for 3 hours.

4.4.1.2 Evaluation the acid resistant properties of EB@TiO2 using bovine model

For the bovine model, thirty-four freshly collected bovine enamel were used to evaluate the acid resistant properties of EB@TiO_2 (see paper III and IV). The tooth samples were sectioned to a diameter of 5mm x 5mm x 3mm using a diamond cutter under water to avoid overheating. Further details of the sample groups are presented in Paper III and IV.

4.4.1.3 Analyzing the acid resistant properties of EB@TiO_2

The acid-resistant characteristics of EB@TiO_2 were evaluated using a different analytical and microscopic instrument. These are described in the sections below.

4.4.1.3.1 Pressure sensor test

A gas pressure sensor (Vernier LabPro, USA) was used to monitor pressure changes in the pressure (kPa) against time (s) during the reaction of the samples with HCI. Erlenmeyer flask (250 mL) was used as the reaction container during the pressure test. A stopper was inserted into the flask to provide an airtight vessel. This was then connected to plastic tubing attached to a gas pressure sensor (Order Code GPS-BTA). With the aid of an interface system (Vernier LabPro) attached to a computer, the pressure readings were collected and analysed using LoggerPro 3 software (Figure 4-5). Prior to the experiment, 0.5g of each sample was placed in the Erlenmeyer flask, while 25mL of the prepared HCl was used as the acid reactant. All tests were performed in duplicate, and the mean slope (kPa/s) values were used for the statistical analysis.

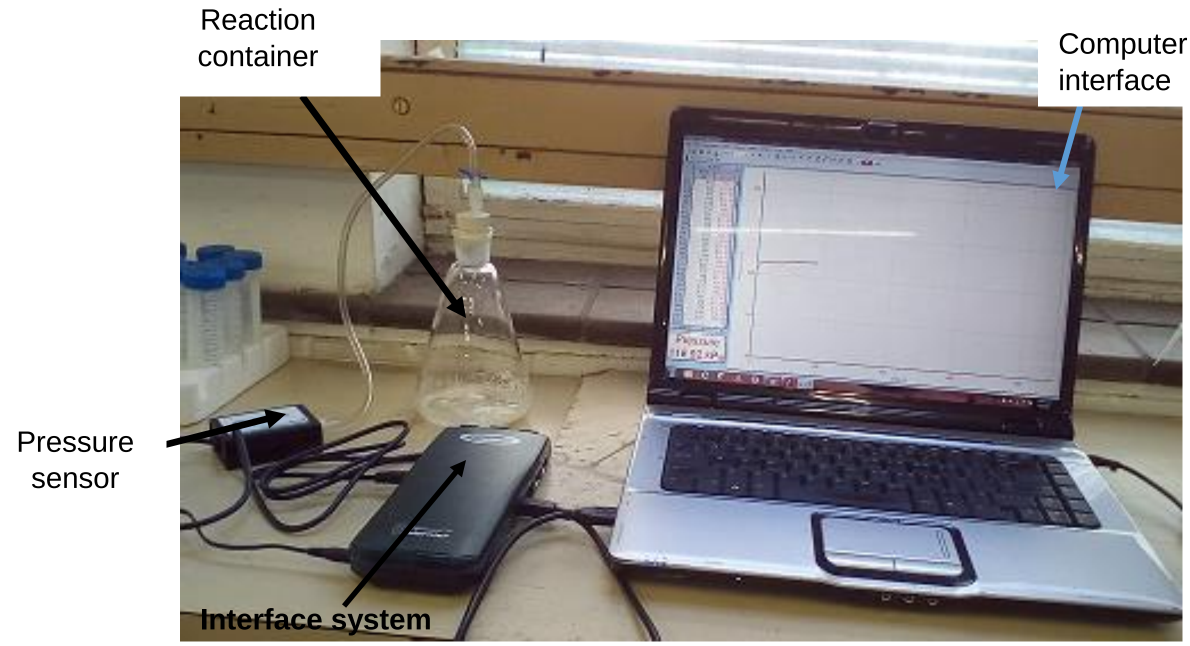

Figure 4- 5: A typical pressure sensor test set-up

4.4.1.3.2 Gas displacement test

A gas-displacement test was used to quantify and measure the rate of reaction by observing the displacement of gas formed during the experiment. This entailed using a cylindrical tube of two cm inner diameter and of 60 cm in height as the gas displacement test set-up (Figure 4-6), a 250 mL Schott bottle, sealed using an airtight stopper, was used for the experiment. A tube was firmly attached through the stopper and connected to a cylindrical tube placed in a reservoir of deionised water. With the aid of a vacuum pump, water from the reservoir was drawn into the cylindrical tube. Prior to the gas displacement test, the initial height of the water in the cylindrical tube was marked using a permanent marker. 0.5g from each sample was placed in the Schott bottle, while 50mL of the prepared 2 M HCl was used as the acid reactant. After 20 minutes, the volume of gas displaced was measured again as the final height of displacement. The amount of gas formed was calculated using equation 1 below.

$$V = \pi r^2 h \quad \text{.......equation 1}$$

Where "V" is the volume of the cylinder, "π" is a constant, approximated as 3.142, "r" the radius of the cylinder and "h" is the displaced height of the cylinder.

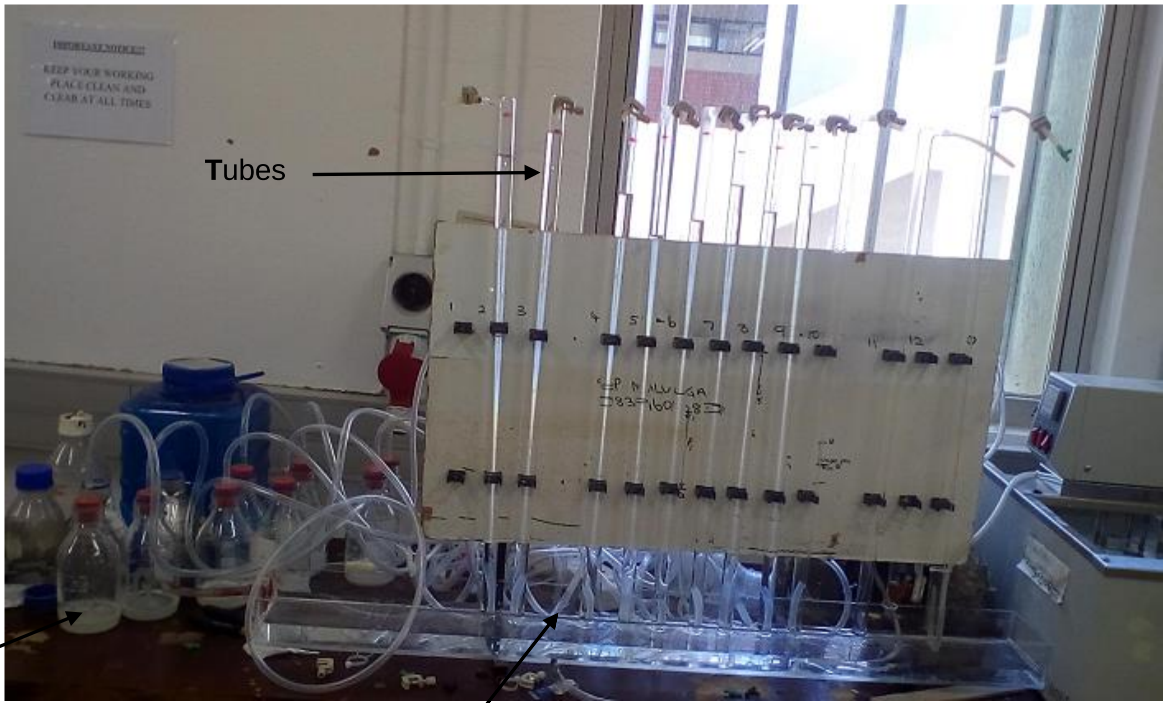

Figure 4- 6: A typical gas-displacement test set-up

4.4.1.3.3 Buffering and pH test

The buffering properties of the EB@TiO_2, and eggshell powder were examined against citric at pH 2, 4, and 5. A stock solution of citric acid was prepared by dissolving 2.1022g of citric monohydrate in a 100mL volumetric flask. Serial dilution was subsequently used to prepare pH 2, 4, and 5, respectively. 1.5g of each sample was placed in a beaker containing 50mL of the prepared citric acid. The solution was constantly agitated at low speed of 600rpm for 30 minutes (Figure 4-7). A pH meter (Starter 300, Ohaus Incorporation USA) equipped with a temperature sensor were constantly used to monitor changes in the pH reading.

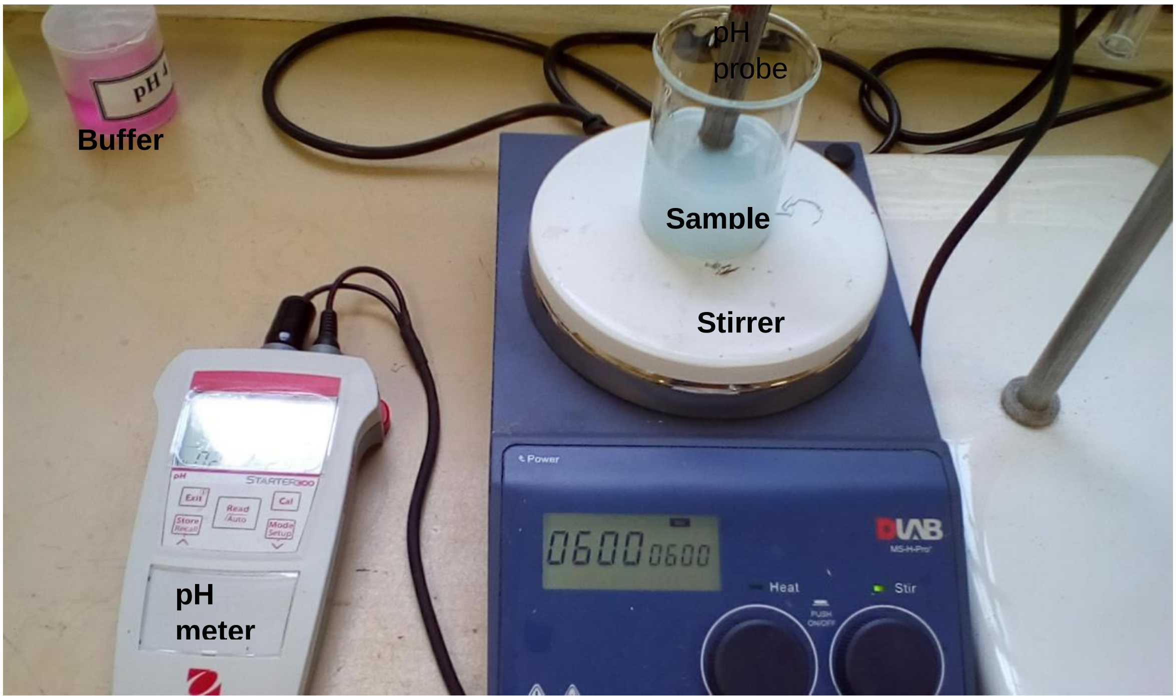

Figure 4- 7: A typical buffering and pH test set-up

4.4.1.3.4 Atomic Force Microscopy (AFM) analysis

AFM (Nanoscope; Bruker), was used to analyze the mean square roughness (R_{rms}) values of the specimens. The instrument was set in a contact mode with a scanning size of 10 µm, and a scan rate of 2.394 Hz. For each specimen, 5 different measurements of the R_{rms} values were made. The average measured R_{rms} values were then used for the statistical analysis (See paper IV).

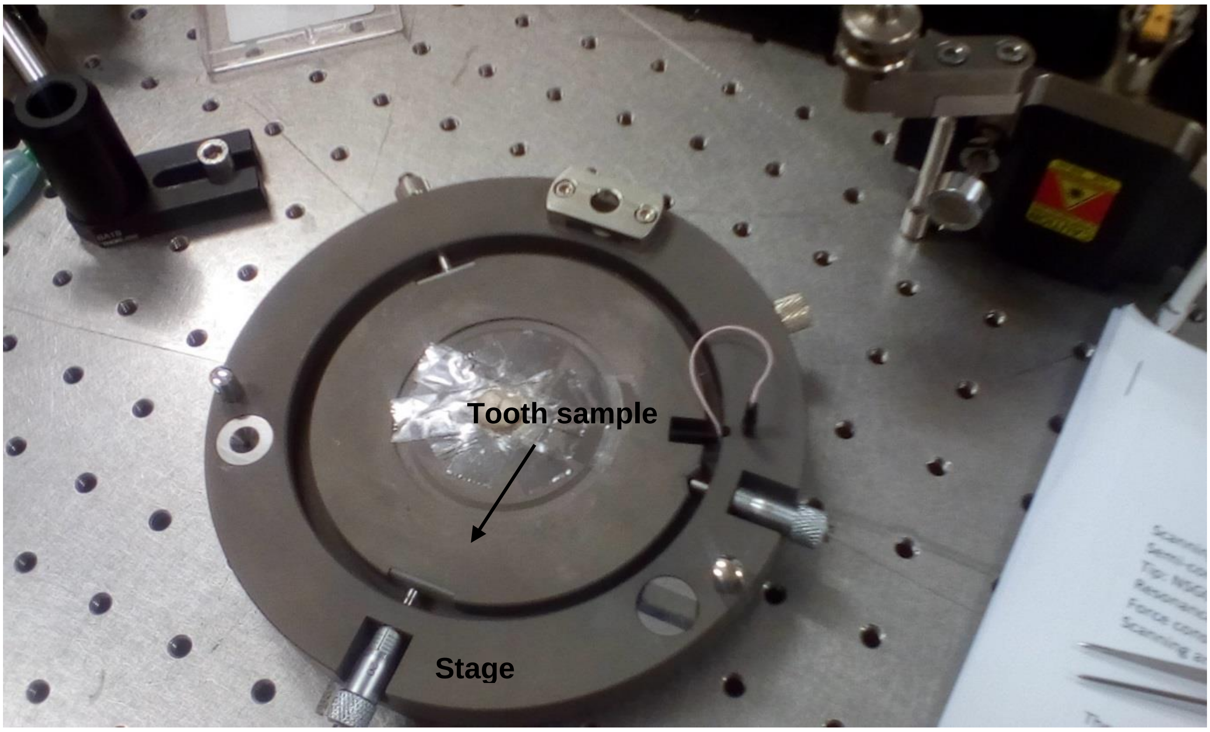

Figure 4- 8: AFM set-up

4.4.1.3.5 Field-Scanning Electron Microscope evaluation of the specimens

The surface of the exposed and unexposed specimens was characterized using Scanning Electron Microscope (FESEM; Carl Zeiss). As a proxy measure, a specimen from each group was analyzed microscopically (See paper III and IV).

4.4.1.3.6 Raman Spectroscopy analysis of specimens

According to Ramakrishnaiah *et al.* (2015) Raman spectrum, particularly from human teeth, reveals changes in the mineral structure as well as the chemical composition which could be useful to study molecular species. In this study, the changes in the mineral content of the specimens were observed using a Raman (Perkin Elmer *precisely* Raman-station 400). The Raman analysis was done on all the samples with the laser power set at 70 mW, exposure every 10 seconds for 3 seconds at a time. Five different measurements were done for each samples and the average was used for statistical analysis (See paper IV).

4.4.2 Assessing the Abrasivity of EB@TiO$_2$

The abrasiveness of EB@TiO$_2$ on tooth enamel were measured in vitro using a simulated brushing protocol (Figure 4-9). Forty-two bovine enamel measuring 5mm x

5mm x 3mm was prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water cooling conditions. Thereafter, enamel disc was embedded in a resin (AMT composite, South Africa). Silicone mold (Silicone rubber mold; Agar scientific) was used to make a mounting base. Before brushing, the samples was kept in artificial saliva for 5min to simulate the formation of the pellicle layer. One half of the tooth was then covered with aluminum tape. Tooth brushing was perform using a powered 1.5v alkaline battery (Oralwise, China) for 2min before rinsing with deionized water. Brushing was performed at room temperature using 100mg of each respective toothpaste (Manuscript III). A tooth brushing protocol load was 200g.

After the brushing protocol, the tape was subsequently removed. The height differences between the covered halves and the brushed halves of the enamel disc were measured using an AFM.

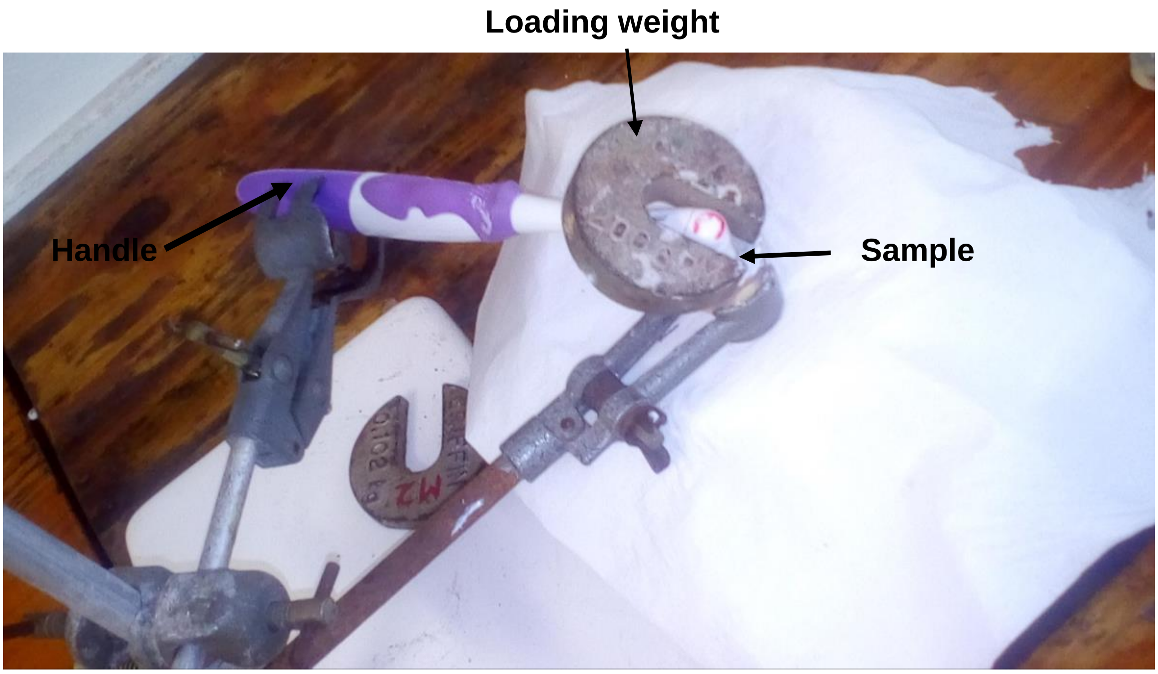

Figure 4- 9: Brushing protocol apparatus set-up.

4.4.3 Assessing the remineralisation and dentin tubule occlusion characteristics of EB@TiO_2

The efficacy of the EB@TiO_2 in occluding open dentinal tubules were assessed *in vitro* using an agitation test as well as the conventional brushing test. These are described in this section.

4.4.3.1 Specimen preparation

Fifty-six freshly extracted bovine-enamel anterior teeth were obtained from a slaughterhouse, South Africa. The collected teeth were subsequently cleaned and disinfected in 10% chloroxylenol solution. Dentin discs measuring 5mm x 5mm x 1mm was prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water cooling conditions. Subsequently, the prepared dentin disc was wet grounded with silicon carbide polishing papers (600-1000 grits) for 60 seconds. Before simulating the sensitive tooth model, the discs were mounted in a resin (AMT composite, South Africa). Silicone mold (Silicone rubber mold; Agar scientific) was used to make a mounting base. A fast setting resin (F160: AMT composite) was mixed in a disposable plastic cup in a 1:1 ratio and poured into the mold. After approximately 2 minutes. The embedded resin was removed from the silicone mold. Thereafter, dentine tubules were opened by soaking the specimens in 1 wt. % citric acid solution for 30 min. The specimens were randomly assigned into different groups (See paper V and VI).

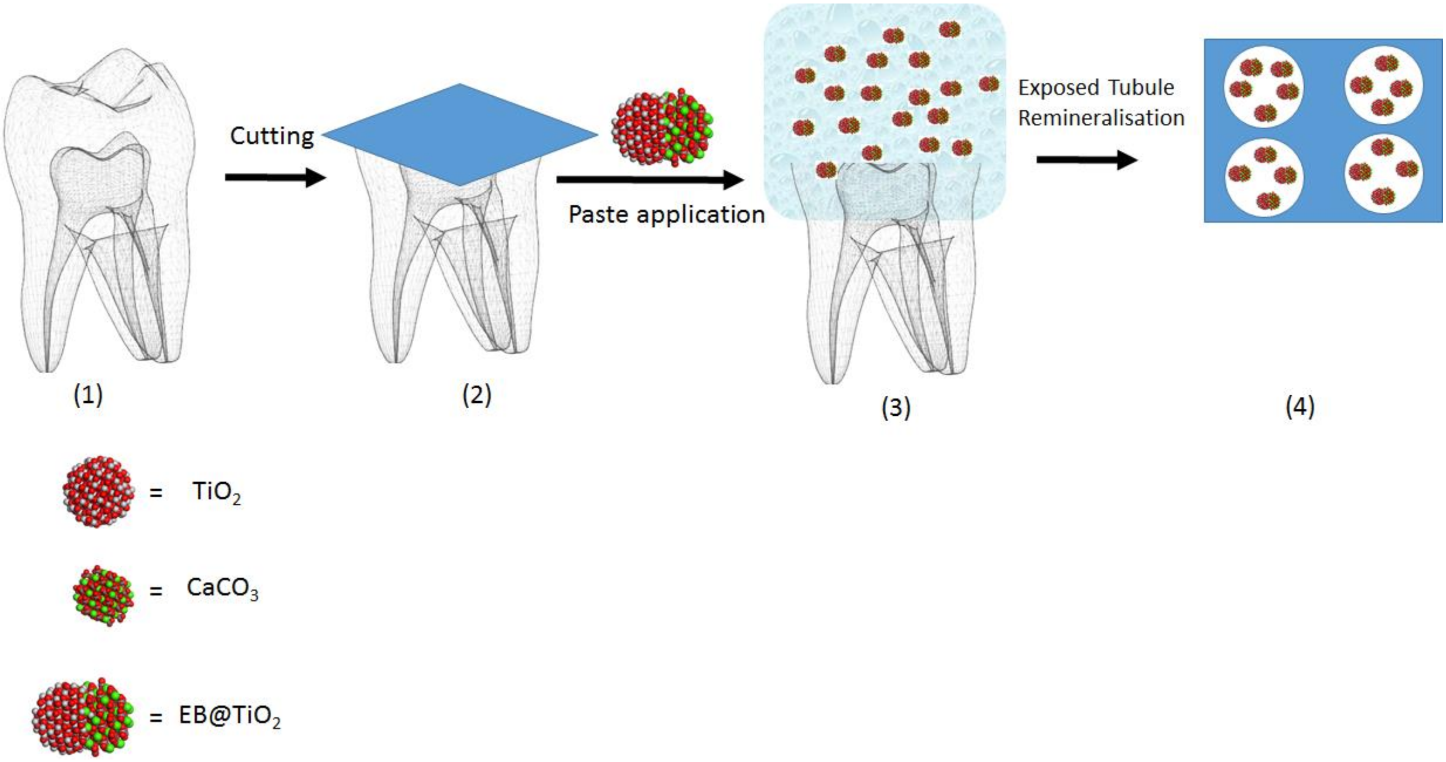

Figure 4- 10: Schematic showing of (1) tooth preparation; (2) EB@TiO_2 application; (3) dentin tubule remineralisation; (4) Observation of dentin surface.

4.4.3.2 Agitation test

The specimens were agitated in a beaker containing 1g of the synthesised EB@TiO_2 and 40mL deionised water for 3 hours. The similar procedure was followed for eggshell powder and Sensodyne paste. The specimens were subsequently rinsed and blot tried. More so, and as a proxy measure, a representative of each sample group were selected to determine the acid resistant characteristics of the treated specimen. This was subsequently exposed to 2wt. % citric acid solution (pH 2) for 5min. After exposure, the specimens were rinsed with deionized water and blot tried.

4.4.3.3 Brushing test

Each specimen from the respective groups (Manuscript II) were brushed twice daily (morning and evening) with a toothbrush powered with 1.5v alkaline battery (Oralwise, China) for 1min and allowed to dry for 30s before rinsing with deionised water.

Brushing was performed at room temperature using 100mg of each respective toothpaste. The slurry of EB@TiO_2 was prepared by mixing 100mg of the powder/200 µL of deionised water. After each brushing protocol, the specimens were immersed in saliva or without saliva as described in Table 4.2. At the end of the seven-day brushing, the treated specimens were exposed to 4% wt. citric acid solution for 2 min to determine the resistance to acidic challenge. After exposure, the specimens were rinsed with deionised water and blot tried.

4.4.3.4 Scanning Electron Microscope evaluation of the occluded specimen

Scanning electron microscope (Field Emission-Carl Zeiss) operating at controlled atmospheric conditions at 20 kV was used to evaluate the occluded dentine pre- and post-acidic exposure (IV and V), as well as each respective days of brushing (Manuscript I). Prior to SEM observation, the surface was coated with a thin, electric conductive gold film to prevent the build-up of electrostatic charge. In addition, the ratios of occluded tubules were further computed using ImageJ software (National Institute of Health USA, http://imagej.nih.gov./ij). This was calculated by dividing the area of occluded tubules by the total tubules area using x 3000 magnification images.

4.4.4 Validating the remineralisation characteristics of EB@TiO_2 using logistic equation to make prediction

4.4.4.1 Model Description

The mathematical model considers that the size of dentin tubules (S), the amount of calcium and phosphates deposits (A) influence significantly the time (t) needed to completely and effectively occlude the dentin tubules.

4.4.4.2 Logistic model

The following logistic equation model was proposed:

$$\frac{dX}{dt} = r_x X \left(1 - \frac{X}{K_x}\right), \qquad (2)$$

where X is the percentage of tubules occluded, r_x the rate at which X is being occluded, K_x the maximum value of X and t is the time to complete remineralisation of dentin tubules. Unless otherwise stated, we will take $K_x = 100\ \%$ since it is the maximum value of X.

4.4.4.3 Analytical solution to the model

By using the method of solving first order ordinary differential equation (separation of variable method) we obtain that the analytical solution of model equation 3 is:

$$X(t) = \frac{K_x\, C\, e^{r_x t}}{1 + C\, e^{r_x t}} \qquad (3)$$

where $C = \frac{X(0)}{K_x - X(0)}$ and $X(0)$ is $X(t)$ at t = 0 (i.e., initial value of X). Further analyses on model (2) show that the model has two equilibrium points: the trivial equilibrium points $X^0 = 0$ and the positive equilibrium $X^* = K_x$. Conducting stability analysis about the two equilibrium states show that the positive equilibrium $X^* = K_x$ is globally stable. This is easily established as $X(t) \rightarrow X^* = K_x$ as $t \rightarrow \infty$. This result shows that it is possible for $X(t)$ to increase to the carrying capacity K_x. On the other hand, the trivial equilibrium point $X^0 = 0$ is unstable. This shows that it will be difficult for $X(t)$ to decrease to zero.

4.4.4.4 Model fitting and parameter estimation

A model fitting and parameter estimation was conducted using our model to fit real data for the three samples (EB@TiO_2, Colgate Pro-relief, Sensodyne repair) for the two cases: with saliva and without saliva. The aim of these analyses is to show that the model we considered can be used to study as well as make future predictions on these samples. For the model fitting, we take the carrying capacity (K_X) as 100% while the growth rate is estimated from the model fittings for all the samples. The model fittings were carried out using the built-in MATLAB Least-squares fitting routine fmincon in the optimisation toolbox.

4.5 Experimental data analysis

Generally, statistics are categories into two namely; inferential and descriptive statistics. In this study, both descriptive and inferential statistics were used to analyse the experimental data. According to Lind, Mason and Marchal (2002), descriptive statistics are typically used to summarised and or organise a set of quantitative data. Whilst descriptive statistics makes no inference or predictions they are, however, useful in visualizing or summarising a set of experimental data. Moreover, it has been suggested in the literature (Salkied 2007; Field 2009) that Univariate, Bivariate and

Multivariate analysis are most appropriate for descriptive statistics. In this study, bar graphs, 2D images, 3D images, and tables were used to present the data.

On the other hands, the inferential statistical analysis makes use of probability laws to learn more about the samples, make inferences, and or draw conclusions about the sample data (Johnson and Christensen 2012). Creswell (2009) reported that inferential statistical tests are ultimately used to test the hypothesis in a study. In testing the hypothesis for this study (Chapter One, Section 1.5), ANOVA was used to compare the mean differences in the experimental sample groups (Paper II, IV, V, Manuscript 1, and II). The previous report by Barnes (2011) noted that the ANOVA is the most appropriate parametric test to identify the mean differences, and for testing any significant differences between three or more variables. Although ANOVA is used in analysing the differences between two or more sample groups, McHugh (2011) pointed out that ANOVA output does not provide any analysis of pairwise differences. In order to compare the difference between each of the five groups, a multivariate statistics (multiple comparisons) using Bonferroni correction were further used to examine pairwise differences in the experimental sample groups (P= 0.05). According to McHugh (2011) Bonferroni test is among the key tests of pairwise differences between sample groups. All analyses were performed using SPSS (Version 25®).

4.5 Validity and Reliability

Validity and reliability is the two most important aspect of research that gives credibility to the study. Although validity can be internal or external, the criterion validity, which is premised on internal validity, was used to ensure the accuracy of the experimental data that was collected and data analysed. Leedy and Ormrod (2005) noted that internal validity permits precise inferences about cause and effect relationships within the data. In this study, the internal validity was ensured by following the SANS 1302 (2008) requirement for the preparation, and testing of desensitising paste. In addition, the experimental data were further validated with the logistic equation model.

With regards to reliability, it is suggested in the literature that the reliability of an experimental research design be determined by the repeatability and reproducibility of the tests (Walker 2011). Slezák and Waczulíková (2011) and Vitek and Kalibera (2011) clarified that repeatability is the variability of the measurement obtained by one person while measuring the same item repeatedly, whilst reproducibility is the variability of the average values obtained by several observers while measuring the same item. In this study, the repeatability and reproducibility was assured by the different number of studies conducted using EB@TiO_2 (Paper II-VI, and Manuscript I-III).

In summary, this chapter has exhaustively explained the research approach and the experimental design strategy carried out in the study. A critical overview of the study will be presented in the next chapter.

Paper I

Onwubu, S.C., Mdluli, P.S., Singh, S. and Bharuth, V. 2018. An in vitro examination on the effectiveness of commercial toothpaste in the prevention of tooth decay using eggshell as the substitute for human tooth. *South African Dental Journal*, 73(7): 446-451**.** http://dx.doi.org/10.17159/2519-0105/2018/v73no7a3.

http://dx.doi.org/10.17159/2519-0105/2018/v73no7a3

An *in vitro* examination on the effectiveness of commercial toothpastes in the prevention of tooth decay, using eggshell as a substitute for human tooth material.

SADJ August 2018, Vol 73 no 7 p446 - p451

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ABSTRACT

Background: Despite improvements in oral health status, dental caries remains a public concern. This study examines the effectiveness of commercial toothpastes in the prevention of tooth decay, using eggshell powder as a substitute for human tooth material.

Methods: Colgate, Aquafresh, Colgate Sensitive, Sensodyne, and Oralwise were tested. An enamel model was simulated by adding eggshell powder to beakers containing dissolved toothpastes. The contents were agitated for eight hours at 800rpm, filtered and oven dried. Scanning Electron Microscope (SEM) and Energy Dispersive Spectroscopy (EDX) were used to characterise the surface morphology and the calcium and phosphate composition of the toothpastes pre- and post- agitation with eggshell powder. Gas-displacement and pressure sensor tests evaluated the rate of reaction between the substitute "tooth enamel" and acids.

Results: EDX analysis confirmed the presence of calcium phosphate ions, while SEM revealed the formation of an enamel-like layer after agitation. Pressure sensor tests confirmed Colgate Sensitive as most effective in protection against acid attacks, with Oralwise least effective.

Conclusions: Eggshell can be used as a substitute for the human tooth in an *in vitro* experiment. All the tested brands of toothpaste effectively reduced the acid reaction, which would contribute to the prevention of tooth decay.

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ACRONYMS

SEM: Scanning Electron Microscope
EDX: Energy Dispersive Spectroscopy

Keywords: eggshell powder, toothpaste, dental caries, tooth enamel

INTRODUCTION

Dental caries, also known as tooth decay, is a common bacterial disease characterised by demineralisation and destruction of hard tissues, usually by the production of acid by bacterial fermentation of undisturbed food debris which has accumulated on the tooth surface.[1-3] Despite the global improvement in oral health status, Inetianbor et al.[4] point out that dental caries remains one of the most chronic and prevalent infectious diseases in the world.[1,5,6] Although dental caries has multifactorial causes,[1] the dietary lifestyles of the individual[2] as well as sub-optimal oral hygiene habits[7] help facilitate the onset of this disease. For instance, it has been reported that the consumption of sweets with high concentrations of glucose, saccharine, or fructose, especially if taken in processed juices, and over a prolonged period play an important role in caries development in children.[8]

Dental caries occurs as a result of the metabolism of bacteria lodging in plaque attached to the tooth, and hence toothpaste and tooth brushing should help reduce the adherence of these microorganisms within the plaque biofilm in the mouth. This is supported by some clinical studies[9,10] which have shown that regular tooth brushing with well-formulated fluoride toothpaste can reduce the incidence of dental caries. Significantly, the dental health organisation in Nigeria has advocated preventive and prophylactic measures in the management of dental caries through regular hygiene and dietary modifications.[4] The increased availability and consumption of soft drinks, fruit juices, and sports drinks,[11] however, make it difficult for individuals to alter their dietary habits. As such, promoting good oral habits through regular brushing with the use of toothpaste may become the most viable option for the oral health care provider in the management of dental caries. Brushing of teeth using toothpaste is recognised as the most commonly practiced form of oral hygiene in most countries.[12] Toothpaste serves as an abrasive which helps in removing dental plaque and food particles from the teeth,[13] as well as assisting in suppressing halitosis and releasing active ingredients, mainly fluoride.[14] Goldman, Yee (15) Strong arguments have been presented that toothpaste is the only realistic fluoride strategy in many low-income countries where lack of infrastructure renders fluoridation of water or salt not feasible.[15] In recent years, the oral health care market has witnessed a boom of different brands of toothpaste, with some claiming to be more effective in protection against acid attack and the prevention of dental caries. But just how effective they actually are remains a question since the continued prevalence of dental caries is still a public health concern.[3] Thus, it is desirable to evaluate the claims that these commercial toothpastes completely prevent the acid attacks that result in caries.

Previous studies suggested that bovine and enamel models be used when examining the effectiveness of commercial toothpaste against acidic attacks.[16,17] Also considered as the sole test model was the dissolution of hydroxyapatite (HA).[18,19] It is generally well known that tooth enamel consists mostly of calcium hydroxyapatite with a molecular formula of $Ca_{10}(PO_4)_6(OH)_2$.[20] Although hydroxyapatite is a hard and resistant compound, acid (H+) that is produced, especially after a high–sugar meal, attacks the apatite causing the enamel to dissolve.[20,21] In this *in vitro* study, eggshells were used as a substitute for human teeth in the examination of factors associated with tooth decay. Eggshells are natural structures composed of both inorganic and organic components such as ~ 97% of calcium carbonates in the form of calcite; 1% magnesium carbonate; 1% apatite ($Ca_3(PO_4)_2$; and ~ 5% of organic matter.[22,23] Owing to this unique chemical composition, eggshells have been used either directly or indirectly as a bone substitute in maxillo-facial surgery,[24] and as source of hydroxyapatite in bone regeneration.[25] To our knowledge, there is limited research on the use of eggshells in examining the effectiveness of commercial toothpaste in the prevention of dental caries. This *in vitro* study was therefore designed to test that premise. It also aimed to evaluate whether commercial toothpaste can slow down the rate of reaction between dental enamel and acids.

MATERIALS AND METHODS

Five different brands of toothpaste were bought from a popular shopping mall located in Durban (South Africa). These toothpastes included: Sensodyne, Colgate Sensitive toothpaste, Aquafresh, Oral-wise and Colgate. The composition, active ingredients and manufacturers of the toothpaste are shown in Table 1. The listed brands of toothpaste were selected on the criteria that they: (1) contained no whitening or herbal formulation that may react with acids and: (2) did contain fluoride in their formulations.

Table 1: Brand, composition, and manufacturer of toothpastes

Products	Composition as indicated in the labeling	Manufacturer
Colgate	Sodium monofluorophosphates, Calcium carbonates, Aqua, Sorbitol, Sodium lauryl sulfate, Aroma, Cellulose gum, Sodium bicarbonate, Tetrasodium pyrophosphate, Benzyl alcohol, Sodium saccharine, Sodium hydroxide, limonene.	Colgate-Palmolive Co.
Oralwise	Sorbitol, Hydrated silica, Deionized water, Propylene, Glycol, Sodium lauryl sulphate, Essence, Cellulose gum, Carrageenan, Sodium saccharin, Sodium benzoate, Tetrasodium pyrophosphate, Sodium fluoride, Titanium dioxide.	Shoprite Checkers (Pty) Ltd.
Colgate Sensitive	Aqua, Gycerin, Hydrated silica, Sorbitol, Potassium nitrate, PEG-12, Tetrapotassium pyrophosphate, Sodium lauryl sulphate, Zinc citrate, PVM/MA copolymer, Aroma, Potassium hydroxide, Xanthan gum, Cellulose gum, Cocamidopropyl betaine, Sodium fluoride, Sodium saccharin, Eugenol, and Limonene	Colgate-Palmolive Co.
Sensodyne	Aqua, Sorbitol, Hydrated silica, Glycerin, Potassium Nitrate, Cocamidopropyl betaine, Aroma, Xanthan gum, Titanium dioxide, Sodium fluoride, Sodium saccharin, Sodium hydroxide, Sucralose, and Limonene	GlaxoSmithKline
Aquafresh	Aqua, Hydrated silica, Sorbitol, Glycerin, Sodium lauryl sulphate, Xanthan gum, Aroma, Titanium dioxide, PEG-6/PEG-8, Sodium fluoride, Sodium saccharin, Carrageenan, and Limonene	GlaxoSmithKline

Preparation of the eggshell powder

Eggshells collected from food outlets were washed to remove impurities, disinfected by immersion in a diluted solution of household sodium hypochlorite for six hours and then vacuum dried for ± 6-9 minutes at 250°C. Thereafter, 20g of the eggshell was placed in a 250ml stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10mm diameter and dry-milled in a planetary ball mill (Retsch® PM 100) at 400 rpm for 20 minutes. The collected powder was then sieved to a particle size of ≤ 25µm using a mechanical sieving machine (Retsch AS 200, Germany).

Simulating tooth using eggshell powder

Three grams of each brand of the toothpastes listed in Table 1 were dissolved in a beaker containing 100 cm^3 deionised water by constant agitation using a magnetic stirrer at 800 rpm for 20 minutes. One gram of the prepared eggshell powder was added to the beaker containing the dissolved toothpaste and the contents agitated for eight hours at a speed of 800 rpm. The mixtures were then filtered and subsequently oven dried at 60 degrees for three hours. Six sample groups were used in this experiment, five containing eggshell powder and dissolved toothpaste (test groups), while the sixth, eggshell powder dissolved in 100 cm^3 deionised water, was used as the control group. Energy dispersive x-ray spectroscopy in conjunction with a scanning electron microscope (Field Emission-Carl Zeiss), operating at controlled atmospheric conditions at 20 kV, were used to determine the elemental composition of the samples and to examine the surface morphology. Prior to SEM observation, the surface was coated with a thin, electric conductive gold film to prevent a build-up of electrostatic charge. As a proxy measure, samples of pre and post agitation of eggshell powder with toothpaste were characterised to establish the formation of a calcium hydroxyapatite layer.

Methods of evaluating the effectiveness of the toothpaste in prevention of tooth decay

The effectiveness of the commercial toothpaste against acid attack was evaluated using 2 M hydrochloric acid. This was prepared by diluting 9.6 mL of acid with deionised water in a 500 mL volumetric flask.

Gas displacement test

A gas-displacement test was used to quantify and measure the rate of reaction by observing the displacement of gas formed during the experiment. This entailed using a cylindrical tube of two cm inner diameter and of 60 cm in height as the gas displacement test set-up. As illustrated in Figure 1, a 250 mL Schott bottle, sealed using an airtight stopper, was used for the experiment. A tube was firmly attached through the stopper and connected to a cylindrical tube placed in a reservoir of deionised water. With the aid of a vacuum pump, water from the reservoir was drawn into the cylindrical tube. Prior to the gas displacement test, the initial height of the water in the cylindrical tube was marked using a permanent marker. 0.5g from each samples were placed in the Schott bottle, while 50mL of the prepared 2 M HCl was used as the acid reactant. After 20 minutes, the volume of gas displaced was measured again as the final height of displacement. The amount of gas formed was calculated using equation 1 below.

$V=\pi r^2 h$……………………………………………equation 1

Where "V" is the volume of the cylinder, "π" is a constant, approximated as 3.142, "r" the radius of the cylinder and "h" is the displaced height of the cylinder.

Figure 1: A typical gas-displacement test set-up

Pressure sensor test

A gas pressure sensor was used to monitor pressure changes (kPa) against time (s) during the reaction of the toothpastes samples with 2 M HCl acid. As shown in Figure 2, an Erlenmeyer flask (250 mL) served as the reaction container during the pressure test, with 0.5g of each sample being placed sequentially in the flask, while 25mL of the prepared 2 M HCl was used as the acid reactant. A stopper fitted with plastic tubing was inserted into the flask to provide an airtight container. The tubing lead to a gas pressure sensor (Order

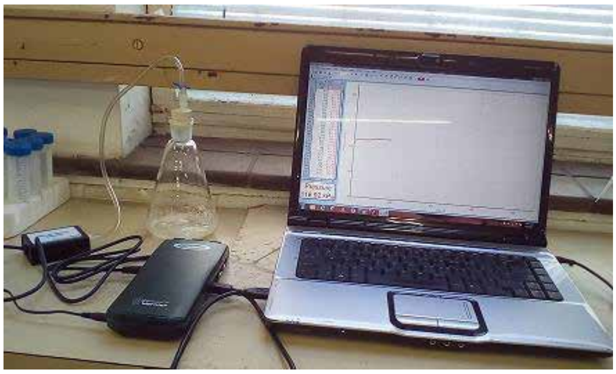

Figure 2: A typical pressure sensor test set-up

Code GPS-BTA). With the aid of an interface system (Vernier LabPro) attached to a computer, the pressure readings were collected and analysed using LoggerPro 3 software.

RESULTS

Characterisation of eggshell powder tooth substitute model

Table 2 illustrates the elemental composition of the various toothpastes brands pre and post agitation with eggshell powder. Significant differences in the amounts of calcium and phosphorus content in the brands of toothpaste were observed between pre and post agitation. In particular, excluding Colgate Sensitive brand of toothpastes, there was a noticeable increase in phosphorus content in the toothpaste samples after agitation with eggshell powder. All the brands of toothpaste containing hydrated silica (Table 1) recorded the presence of calcium after agitation.

Table 2: Elemental composition of toothpastes samples showing calcium (Ca), phosphorus (P), and oxygen (O) content before and after agitation with eggshell powder as determined by EDX analysis

Sample Group	Pre agitation			Post agitation		
	Ca	P	O	Ca	P	O
Colgate	34.04	0.02	50.03	25.38	0.09	52.47
Oralwise	0	0.01	45.93	31.6	0.22	41.54
Colgate sensitive	0	0.53	48.94	22.12	0.37	54.05
Sensodyne	0	0.06	62.41	34.99	0.31	42.51
Aquafresh	0	0	55	9.84	0.04	49.21

The SEM micrograph shown in Figure 4-7 revealed differences in the surface morphology pre and post agitation with eggshell powder. For the Colgate brand of toothpaste shown in Figure 3, for example, it can be observed that the surface morphology of toothpaste

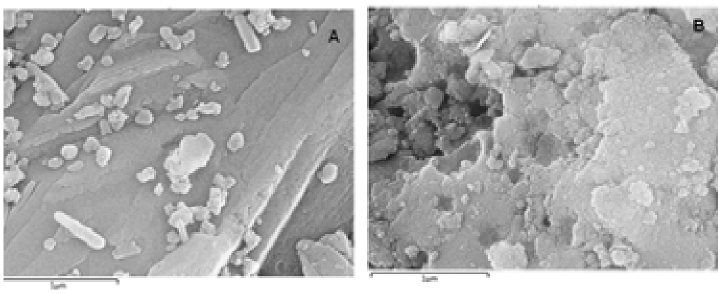

Figure 3: SEM micrographs of Colgate toothpaste samples (A) pre agitation; (B) post agitation with eggshell powder.

became more compact and dense post agitation with eggshell powder (Figure 3B), compared with the brand pre agitation (Figure 3A). Similar patterns and compactness in the toothpaste after agitation were observed for the other brands of toothpaste (Figures 4 to 7).

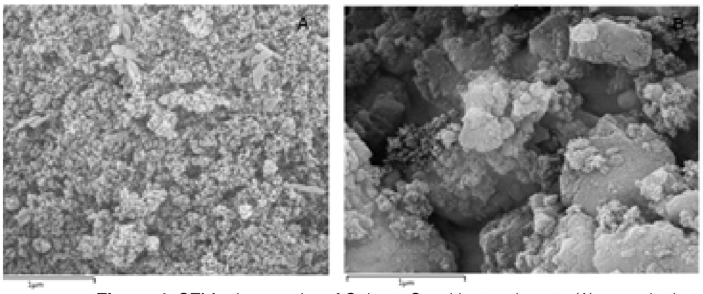

Figure 4: SEM micrographs of Colgate Sensitive toothpaste (A) pre agitation; (B) post agitation with eggshell powder.

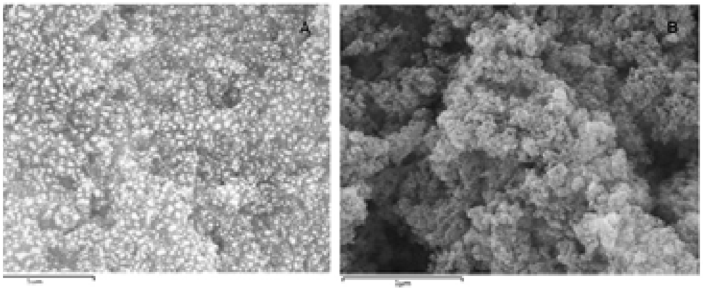

Figure 5: SEM micrographs of Sensodyne toothpaste (A) pre agitation; (B) post agitation with eggshell powder.

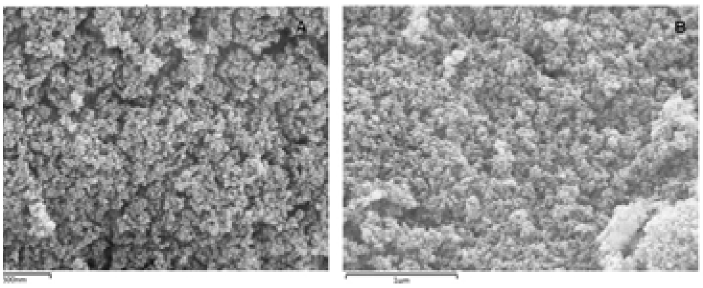

Figure 6: SEM micrographs of Oralwise toothpaste (A) pre agitation; (B) post agitation with eggshell powder.

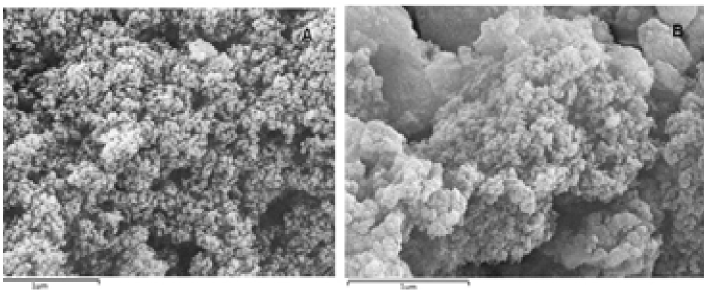

Figure 7: SEM micrographs of Aquafresh toothpaste (A) pre agitation; (B) post agitation with eggshell powder

Acid resistance properties of commercial toothpastes

Figure 8 illustrates the results of the gas-displacement test from the various brands of toothpastes after reaction with 2 M HCl acids. In contrast to the control sample (eggshell powder dissolved in water), the various brands of toothpastes generated a lesser volume of gas. The amount of gas produced is in accord with the average mean pressure of gas measured in the gas pressure tests (Figure 9). Overall, it can be gathered that the Aquafresh brand of toothpastes generated the least amount of gas in reaction to the acid when compared with the rest of the brands.

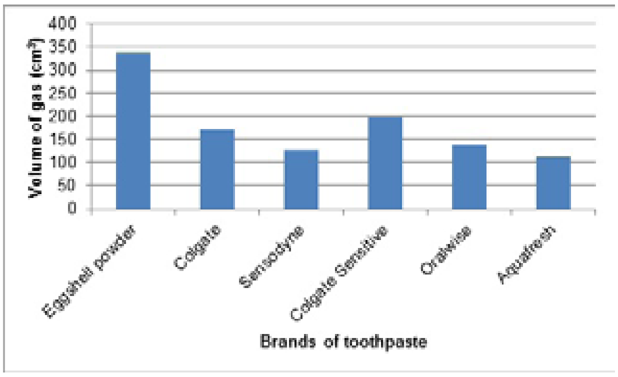

Figure 8: Showing mean gas displacement of the brands of toothpastes reacted with 2 M HCl acids.

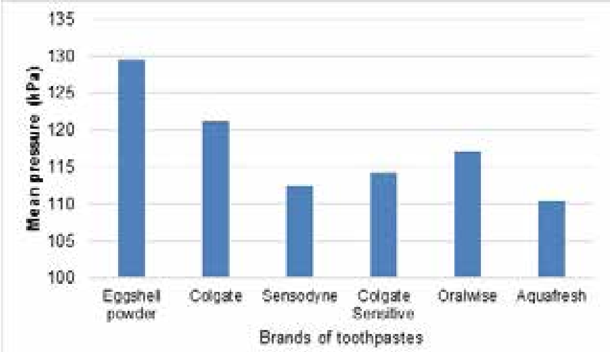

Figure 9: Showing mean gas pressure produced during the reaction of 2 M HCl acids with the brands of toothpastes.

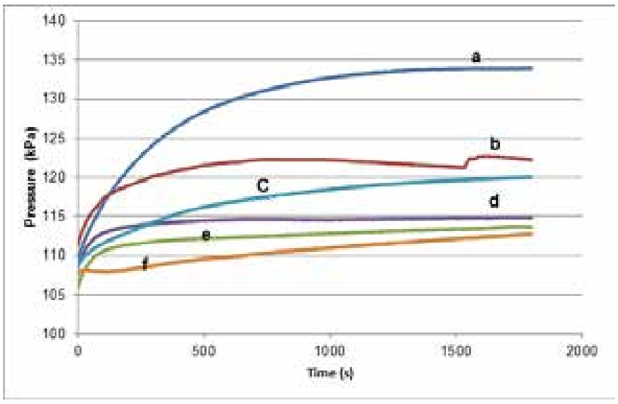

Figure 10: The amount of gas produced during acid reaction with (a) eggshell powder; (b) Colgate; (c) Aquafresh; (d) Colgate Sensitive; (e) Sensodyne; (f) Oralwise through to 1800 seconds.

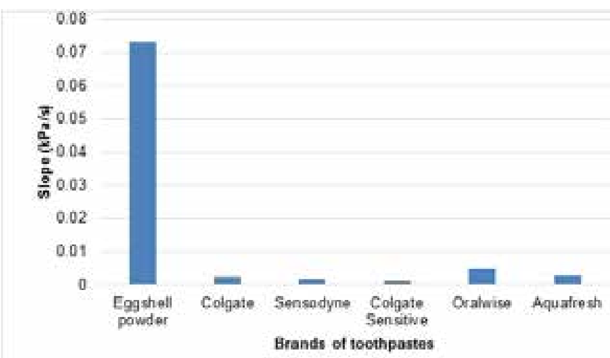

Figure 11: The gas pressure produced during the reaction of 2 M HCl acids with the brands of toothpastes.

The amount of pressure (kPa) generated during the gas test was plotted against time (seconds) (Figure 10). It may be observed that reaction to acid was low in the various brands of toothpaste after 100 seconds. Thereafter, increased acid reaction could be observed. After 500 seconds, all the test samples recorded near plateau-like graph lines suggesting no significant changes were occurring in the quanta of gas pressure generated. The Oralwise sample produced the lowest curve (d), while the control had the widest curve(a). Notwithstanding this, Colgate Sensitive brand of toothpaste had the lowest initial rate of acid reaction, while the eggshell powder (control) had the highest initial rate (Figure 11).

DISCUSSION

Despite general improvements in oral health status, dental caries still remains a significant public concern posing a grave challenge for the oral health care provider in mitigation and prevention. Published literature suggests that brushing regularly with well formulated fluoride toothpastes can prevent the onset of this disease.[9,10,26,27] In the present *in vitro* study, eggshell powder was used as a substitute for human tooth material to verify the effectiveness of five commercially available toothpastes in the prevention of dental caries. SEM and EDX were used to study the surface morphology and the calcium and phosphate composition of the brands of toothpastes before and after agitation with eggshell powder.

Calcium phosphate is regarded as being fundamental for the formation of teeth.[28] Based on the EDX elemental analysis (Table 2), calcium phosphate ions were evidently present post agitation with eggshell powder. Notably, the SEM micrographs of the tested brands of toothpastes (Figure 4-7) were able to show net differences pre and post agitation with eggshell powder, which suggests the formation of enamel like hydroxyapatite layer in all brands of toothpastes. In light of the differences in the surface morphology as well as the elemental composition of the tested brands of toothpastes, it can be inferred that eggshell will be suitable as a human tooth substitute for *in vitro* assessment of erosive attack. This is in agreement with Haghgoo, Mehran (29) that eggshell powder could be used as alternative for hydroxyapatite since it contain calcium, phosphorus and other mineral element.

Furthermore, effectiveness the toothpaste against HCl acid attacks was evaluated using both gas-displacement and pressure sensor tests. The data generated from the gas tests suggest that Aquafresh brand of toothpaste is more likely to produce the less volume of gas when reacted with acids (Figure 8 and 9). However, the volume of gas generated from the test samples is not indicative of the effectiveness of the brands of toothpaste in the prevention of acid attacks. As reported in Figure 11, Colgate Sensitive had the lowest initial rate of acid attack, followed by Sensodyne, hence their ability to prevent acid reaction with the tooth that causes dental caries. Overall, all the tested brands of toothpaste tend to be effective in the prevention of dental caries by reducing the reaction of the tooth enamel to HCl acid exposure.

Importantly, and in respect to time of protection, clear differences in the prevention of acid attacks were observed between the tested brands of toothpastes and control (eggshell powder dissolved in water). From the graphical results shown in Figure 10, as expected, the various brands of toothpastes resulted in less acid reaction than the control. For instance, the tested toothpastes provided greatest protection to the tooth surface in less than 2 minutes (100 seconds) after acid attacks. In contrast, more acidic reaction could be observed for the control sample even after 8-10 minutes (above 500 seconds) exposure to the acids. This suggests that human tooth can react with acid more easily without any protection offered by toothpastes. Hence, it can be gathered that the composition of the tested toothpastes, particularly the fluoride content is responsible for the protection of the tooth against acid attacks. Kallahalli, Sanjay (30) reported that fluorides are abundantly used in many oral health products including toothpastes and mouth rinses as they help in caries prevention by reducing dental caries between 30 and 70% compared with no fluoride therapy. The findings from this study therefore confirmed the effectiveness of fluoride-based toothpastes in the prevention of dental caries.

The protective effect against erosion observed for Colgate Sensitive and Sensodyne, for example, is in line with previous studies. Lombardini, Ceci[31] reported that Colgate Sensitive Pro Relief was more effective than Sensodyne in the protection of enamel against acid attacks. Kato, Lancia,[16] who used bovine enamel, observed that Colgate Sensitive and Sensodyne Original had the best protective effect against erosive acids.

Drawing from the above discussion, it is sufficient to say that the eggshells model was successfully used to evaluate the protective effect of commercial toothpaste against acid attacks. This may be important, particularly in the context of oral health care, as eggshells present a suitable, cheaper, and readily available alternative source to the use of bovine and human enamel models in examining the protective effect of commercial toothpaste. The study suggests that gas displacement and the use of a pressure sensor present a new method of evaluating the rate of reaction between enamel and acids.

LIMITATIONS

The gas displacement and pressure tests cannot measure mineral dissolution and surface changes in the eggshell model. Future research could be directed at an examination of the surface of the eggshell model post acid exposure. This would help explain the mechanisms associated with the protective effect of the tested toothpastes by evaluating the mineral dissolution in the eggshell model.

CONCLUSION

Eggshell powder can be used as a substitute for human tooth in an *in vitro* experiment. Notably, this study conclusively showed that of the test products, Colgate Sensitive is the most effective brand of toothpaste against acid attacks, while Oralwise is the least effective. However, all brands of the tested toothpastes did lower the rate of reaction between HCl acid and eggshell powder tooth substitute, suggesting their ability to protect the tooth from acids that cause dental caries.

Acknowledgment

The authors acknowledge the financial support from the National Research Foundation of South Africa (No. 104824).

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Paper II

Onwubu, S.C., Mdluli, P.S. and Singh, S. 2019. Evaluating the buffering and acid-resistant properties of eggshell-titanium dioxide composite against erosive acids. *Journal of Applied Biomaterial and Functional Material, 1-7***.** https://doi.org/10.1177/2280800018809914

JABFM Journal of Applied Biomaterials & Functional Materials

Original Research Article

Journal of Applied Biomaterials & Functional Materials
January-March: 1–7

DOI: 10.1177/2280800018809914
journals.sagepub.com/home/jbf
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Evaluating the buffering and acid-resistant properties of eggshell–titanium dioxide composite against erosive acids

Stanley Chibuzor Onwubu[1], Phumlane Selby Mdluli[2] and Shenuka Singh[3]

Abstract

Objective: This paper reports on the buffering and acid-resistant properties of a modified eggshell–titanium composite against citric acid attack.

Materials and Methods: Eggshell–titanium EB-TiO_2 was prepared by ball-milling eggshell powder and titanium dioxide. Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), and Transmission Electron Microscopy (TEM) were used to characterize EB-TiO_2. The buffering property against citric acid at pH values of 2, 4, and 5 was measured using a pH meter. Five brands of toothpaste (Colgate, Colgate Sensitive, Aquafresh, Oralwise, and Sensodyne) were used to assess the acid-resistant properties of EB-TiO_2. Enamel models were simulated by dissolving each brand of toothpaste with eggshell (control) and EB-TiO_2. The samples were exposed to citric acid of pH 2. The average slope (kPa/s) was measured using a pressure sensor. An analysis of variance was used to analyze the kPa/s values (α =.05).

Results: The FTIR and XRD analyses suggest the surface modification of EB-TiO_2. The TEM image revealed spherical-shaped particles in EB-TiO_2. The pH test results showed that the buffering properties of eggshell and EB-TiO_2 were comparable. Significant differences were observed in the acid resistance properties of the samples exposed to citric acids ($P < .05$). The Colgate toothpaste infused with eggshell powder had the highest mean kPa/s values, whereas Sensodyne infused with EB-TiO_2 had the lowest kPa/s values.

Conclusion: The salient features of this study indicate that modification of eggshell with titanium dioxide does not affect its carbonate buffering properties. Connecting the kPa/s values to acid-resistant properties, EB-TiO_2 effectively reduces erosive attacks when added to toothpaste.

Keywords

Citric acid, eggshell powder, toothpaste, titanium dioxide

Date received: 17 January 2018; revised: 29 August 2018; accepted: 8 October 2018

Introduction

Enamel erosion is a common occurrence and, under extreme conditions when the enamel is exposed to a highly acidic oral environment, it will result to the dissolution of the enamel surface.[1–3] Previously, enamel erosion, particularly in the cervical area, was mostly reported among elderly people.[4] Several studies[4–7] alleged that a change in diets as well poor oral hygiene have significantly contributed to the frequency of enamel erosion among young people. More so, enamel erosion has become an important issue with the increased consumption of acidic drinks, such as sports drinks, soft drinks, and citric juices.[3,5,8,9] More worrisome is that the afore-mentioned products have a pH value below the critical level for dissolving dental enamel.[3,8] Gambon et al.[10] point out that citric acid, for example, is a complex acid with respect to erosive

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potential. They noted that when the pH is at a lower level, the citric acid releases $[H]^+$ ions that attack the surface enamel, while at higher values of pH, the citrate anion is able to chelate calcium from the enamel surface.

Generally, human tooth enamel is predominantly hydroxyapatite in the form of phosphate ions (PO_4^{3-}) and calcium ions (Ca^{2+}).[7,11] Fejerskov[12] and Featherstone[13] noted that a stable equilibrium exists between the crystalline hydroxyapatite making up the 96% of tooth enamel and the phosphate and calcium ions in saliva. The destabilization of the afore-mentioned equilibrium, particularly when the oral environmental pH drops below a critical level (5.5 for enamel and 6.2 for dentin), may result in the dissolution of tooth mineral (hydroxyapatite) in a process called demineralization.[14] In contrast, with the elevation of the oral environmental pH promoted by the natural buffer capacity of saliva, the mineral gets reincorporated into the tooth through the process of remineralization.[15] While saliva is considered an important biological factor that dictates the intraoral neutralizing effect of acid exposure, Neel et al.[2] stressed that some medication and asthmatic inhalers may reduce the flow of saliva, thereby inducing xerostomia. This, and according to Kargul et al.[16] and Sivasitamparam et al.,[17] decreases the pH of saliva, weakening its overall buffering effect against intrinsic and extrinsic acids. Moreover, the loss of enamel material by erosion is a dynamic process occasioned with periods of demineralization and remineralization; hence, preventive measures against enamel dissolution from acid and potentially permanent damage should be a priority for oral health care providers.[3,4]

Importantly, toothpastes have been considered affective and accessible vehicles to improve enamel resistance against erosive oral environments.[18] In recent years, different ingredients have been incorporated into toothpastes to increase their protective effect against erosive attacks. Among these ingredients, the use of topical fluoride to modify the effects of erosion at the tooth surface is well documented.[8,19–21] Bertassoni et al.[22] and Moretto et al.,[23] in their asserted views, however, highlighted that fluoride toothpastes alone are not capable of completely inhibiting tooth wear. Resonating with them, Moron et al.[15] stressed that conventional fluoride-containing toothpastes lack the capacity to protect sufficiently well against erosive challenge. Further to this, it has been shown at below pH 3 that the protective effect of fluoride is diminished.[24] Consequently, the addition of calcium or calcium-containing material in toothpastes is suggested to have beneficial health effects against erosive attacks.[25] Davis et al.[26] reported that calcium-fortified juices were sufficient to prevent erosion occurring in human tooth enamel. In support of them, Magalhães et al.[27] found that the modification of carbonated fruit drink juice-based drinks with calcium reduced their erosive potential.

Of interest, eggshells are naturally composed of calcium carbonates (~97% in the form of calcite); 1% magnesium carbonates; 1% apatite ($Ca_3(PO_4)_2$; and ~5% organic matter.[28,29] Several studies[30–32] have shown that the calcium constituent in eggshell was similar to that found in bone and teeth, thereby supporting its use as an abrasive cleaner in toothpastes. Furthermore, Cutler[33] suggested that titanium dioxide and calcium carbonate-based abrasive agents when used together effectively reduce tooth sensitivity. Equally significantly, Lin et al.[34] and Tao et al.[35] have shown that the mechanical activation of calcium carbonates together with titanium dioxide improved the acid resistance properties when used in the paper making industry. Despite the benefits associated with the modification of calcium carbonates with titanium dioxide, limited evidence is available on its use in the prevention of erosive attacks. In this paper, we present a new product based on the modification of eggshell powder and titanium dioxide through ball-milling, as a potential ingredient in toothpastes for the protection of erosive attacks.

In recent years, the modification of eggshell waste through ball-milling to obtain a novel material has gained much attention amongst researchers, owing to its environmental friendliness, less use of harmful organic solvents and energy, and its reproducibility, with a high yield under simple and easy operating conditions.[36,37] According to Baláž,[37] ball-milling is a tool of mechanochemistry that combines the solid state approach and mechanical energy input for various desired applications. The author alleged that ball-milling natural material such as eggshells offers the prospect to change their application potential to a new level. Significantly, Battistella et al.[38] advocated for the use of biomaterial obtained from natural sources in dentistry due to its outstanding healing properties, biocompatibility with natural tissues, abundant availability, and low cost. This paper therefore aimed to examine the buffering and acid-resistant properties of a ball-milled eggshell–titanium dioxide (EB-TiO_2) composite against citric acid attack.

The formulated hypothesis was as follows: (1) EB-TiO_2 is a suitable ingredient in toothpaste formulation as it improves acid resistance when added into toothpastes.

Materials and methods

Food grade anatase titanium dioxide (CAS No: 13463677) was purchase from Sigma-Aldrich (Germany). Citric acid monohydrate and lactic acid were supplied by Merck (South Africa). Five different brands (Sensodyne, Colgate sensitive toothpaste, Aquafresh, Oral-wise, and Colgate) of toothpastes were bought from a popular shopping mall located in Durban (South Africa).

Preparation of eggshell–titanium dioxide material

As previously described by Onwubu et al.,[39] eggshells obtained from Durban fast foods were washed with regular detergent to remove impurities. Eggshells were then disinfected by storing the eggshells in a diluted solution of household sodium hypochlorite for 6 hours. Thereafter, eggshells were vacuum dried for ±6–9 minutes at 250°C. Modification of eggshells with titanium dioxide was achieved in two steps. In the first step, eggshells were ball-milled by placing 30 g of the eggshell in a 500 ml stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10 mm diameter, and dry-milled in a planetary ball mill (Retsch ® PM 100) at 400 rpm for 20 min. The low milling time of 20 min allows for the homogenization of the eggshell powder particle sizes.[37] The collected powder was then sieved to a particle size of ⩽25 µm using a mechanical sieving shaker (Retsch AS 200, Germany). Thereafter, the fine eggshell powder obtained in step 1 was modified according to the procedure described by Lin et al.[34] A total of 20 g of the fine eggshell powder was modified by adding 5 g of food grade anatase titanium dioxide (⩽15 µm) and subsequently ball-milled for 200 min to obtain a nanosized eggshell–titanium dioxide material (⩽80 nm). Lin et al.[34] suggest that a new material is capable of forming after ball-milling calcium carbonate-based material with titanium dioxide for above 120 min.

Characterization of EB-TiO_2

Fourier Transform Infrared Spectroscopy analysis. The infrared spectra were measured using a Perkin Elmer Universal ATR spectrometer to identify the functional group constituents of EB-TiO_2. A very small amount of sample was placed in the sample holder. An initial background check was performed before scanning in the range of 400–4500 cm^{-1}.

X-Ray Diffraction analysis. The crystallinity of the modified Eb-TiO_2 was assessed using X-Ray Diffraction (XRD). The XRD diffractometer (PANalytical-Empyrean instrument; Co radiation 1.54056 A°) was calibrated with a voltage of 40 kV, current of 40 mA, and time of 1 s and analyzed between 0° and 90° (2 theta).

Transmission Electron Microscopy analysis. Transmission Electron Microscopy (TEM) was used to observe the particle size, shape, and distribution of EB-TiO_2. Some amounts of the samples were dispersed in 5 ml ethanol and sonicated at 10 kV for 20 min. Then, thin cross-sections of cryo-microtomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids. Analysis was conducted using a transmission electron microscope (TEM-Philips CM 120 model) at 120 kV.

Examining the buffering properties of EB-TiO_2

The buffering properties of the EB-TiO_2 and eggshell powder were examined against citric acid at pH values of 2, 4, and 5. A stock solution of citric acid was prepared by dissolving 2.1022 g of citric monohydrate in a 100 ml volumetric flask. Serial dilution was subsequently used to prepare pH values of 2, 4, and 5, respectively. A total of 1.5 g of each sample was placed in a beaker containing 50 ml of the prepared citric acid. The solution was constantly agitated at the low speed of 600 rpm for 30 min. A pH meter (Starter 300, Ohaus Incorporation USA) equipped with a temperature sensor was constantly used to monitor changes in the pH reading.

Preparation of the simulated enamel model

Each brand of toothpaste was used to assess the acid-resistant properties of EB-TiO_2. The enamel model was simulated by dissolving each brand of toothpaste with eggshell (control) and EB-TiO_2. This entails dissolved the substance in a beaker containing 100 cm^3 deionized water. A total of 1 g of the prepared eggshell powders and EB-TiO_2 was added to the beaker containing the dissolved toothpastes. These were further agitated for 8 h at a speed of 800 rpm. The mixtures were filtered and oven dried at 60 degrees for 3 h. The samples were exposed to citric acid of pH 2 for 30 min.

Evaluation of acid-resistant properties. The acid-resistant properties of the prepared samples were analyzed using a gas pressure sensor (Order Code GPS-BTA). Using an Erlenmeyer flask (250 ml) as the reaction vessel, the pressure sensor was connected to an interface system (Vernier LabPro, USA). The mean slope (kPa/s) was recorded using LoggerPro 3 software. Prior to the experiment, 0.5 g of each sample was placed in the Erlenmeyer flask, while 25 ml of the prepared citric acid was used as the reactant. All tests were performed in duplicate, and the mean slope (kPa/s) values were used for the statistical analysis.

Statistical analysis

Using a statistical package (IBM SPSS Statistics v 24; IBM Corp), the mean kPa/s values were evaluated and compared with one-way analysis of variance (ANOVA).

Results

Characterization of EB-TiO_2

The Fourier Transform Infrared Spectroscopy (FTIR) spectra of eggshell powder and EB-TiO2 are given in Figure 1. As shown in Figure 1(a), the absorption peaks of the carbonate structures observed at 1411, and 711, and 873 cm^{-1} are attributed to the presence of calcite found in eggshell.[39] In contrast, the FTIR spectra in Figure 1(b) show a broad band below 800 cm^{-1}, which corresponds to the Ti-o-Ti vibrations of the titanium dioxide.

The XRD pattern of eggshell powder and EB-TiO2 showing characteristic diffraction peaks with diffraction angles is given in Figure 2. Consistent with Onwubu et al.,[39] the characteristic peak marked around 34.5° indicates the presence of calcite (Figure 2(a)). Furthermore, the XRD pattern of

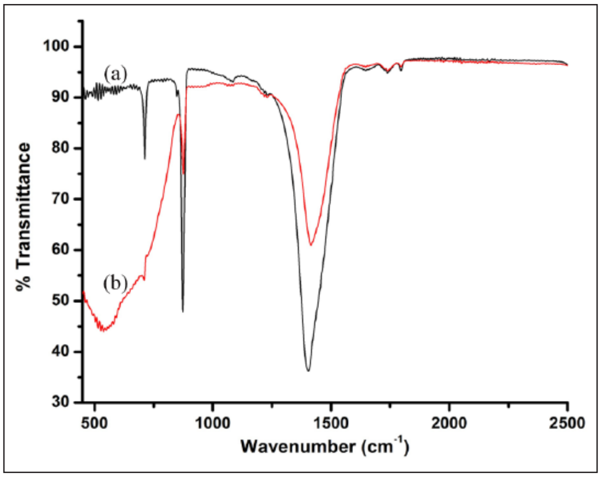

Figure 1. Fourier Transform Infrared Spectroscopy spectra of (a) eggshell powder and (b) EB-TiO_2.

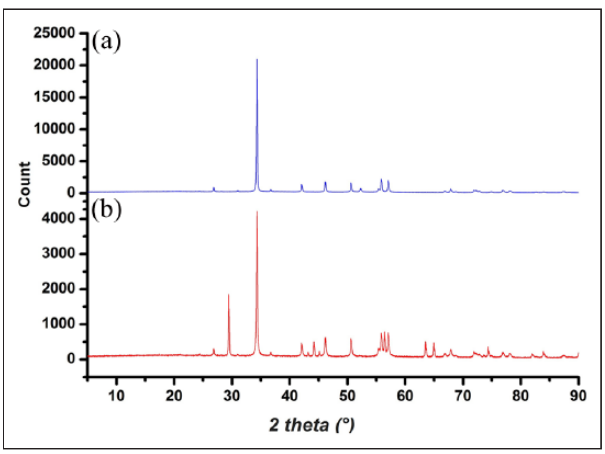

Figure 2. X-ray Diffraction pattern of (a) eggshell powder and (b) EB-TiO_2.

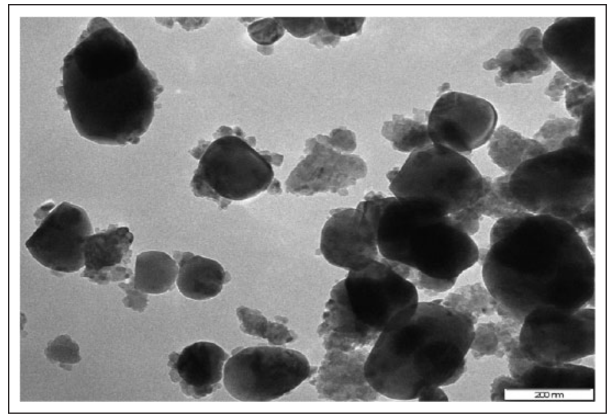

Figure 3. Transmission Electron Microscopy images of EB-TiO_2 showing particle shapes.

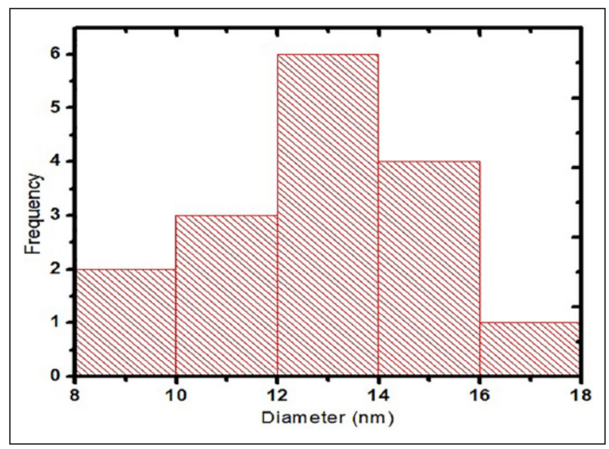

Figure 4. Histogram of EB-TiO_2 showing the average mean particle sizes.

EB-TIO_2 given in Figure 2(b) reveals a characteristic diffraction peak with values lying at 2 theta (29.5°). This relates to the anatase phase of titanium dioxide and is confirmed by the International Centre for Diffraction Data (ICDD Ref: 98-009-6946). Furthermore, the shape, intensity, and location of the EB-TiO_2 peaks suggest that TiO_2 is deposited on the surface of the $CaCO_3$ constituents of eggshell powder. The above assertion further supports the work of Tao et al.,[35] who observed the deposition of pure TiO_2 on the surface of $CaCO_3$ at diffraction peaks with values around 27.5°.

Equally important, and contrary to the report by Baláž et al.[40] that ball-milling induces phase transformation of the calcite in eggshell to aragonite, the XRD pattern in Figure 2 suggests the absence of phase transformation. The differences in these studies could, however, be attributed to the milling conditions. According to Baláž,[37] ball-milling eggshell for 240–360 min causes phase transformation in the eggshell powder from calcite to aragonite and vice versa. A similar factor may have contributed to the absence of phase transformation of EB-TiO_2, as the composite was ball-milled for 200 min.

The TEM micrograph of EB-TiO_2 is given in Figure 3(a). The presence of nonhomogeneous particles with some agglomeration was observed. In addition, the TEM revealed that irregular-shaped particles coexist together with spherical-shaped particles. The presence of the irregular-shaped particles could be attributed to the calcite shape of the eggshell powder, whilst the spherical-shaped particles typified the presence of TiO_2.

The particle distribution of EB-TiO_2 analyzed using ImageJ (National Institute of Health USA) reveals an average mean particle size of around 13 nm (Figure 4).

Buffering and acid-resistant analysis

Figure 5 illustrates the buffering properties of the tested samples against citric acids with different pH values of 2, 4, and 5, respectively. The results suggest that the buffering

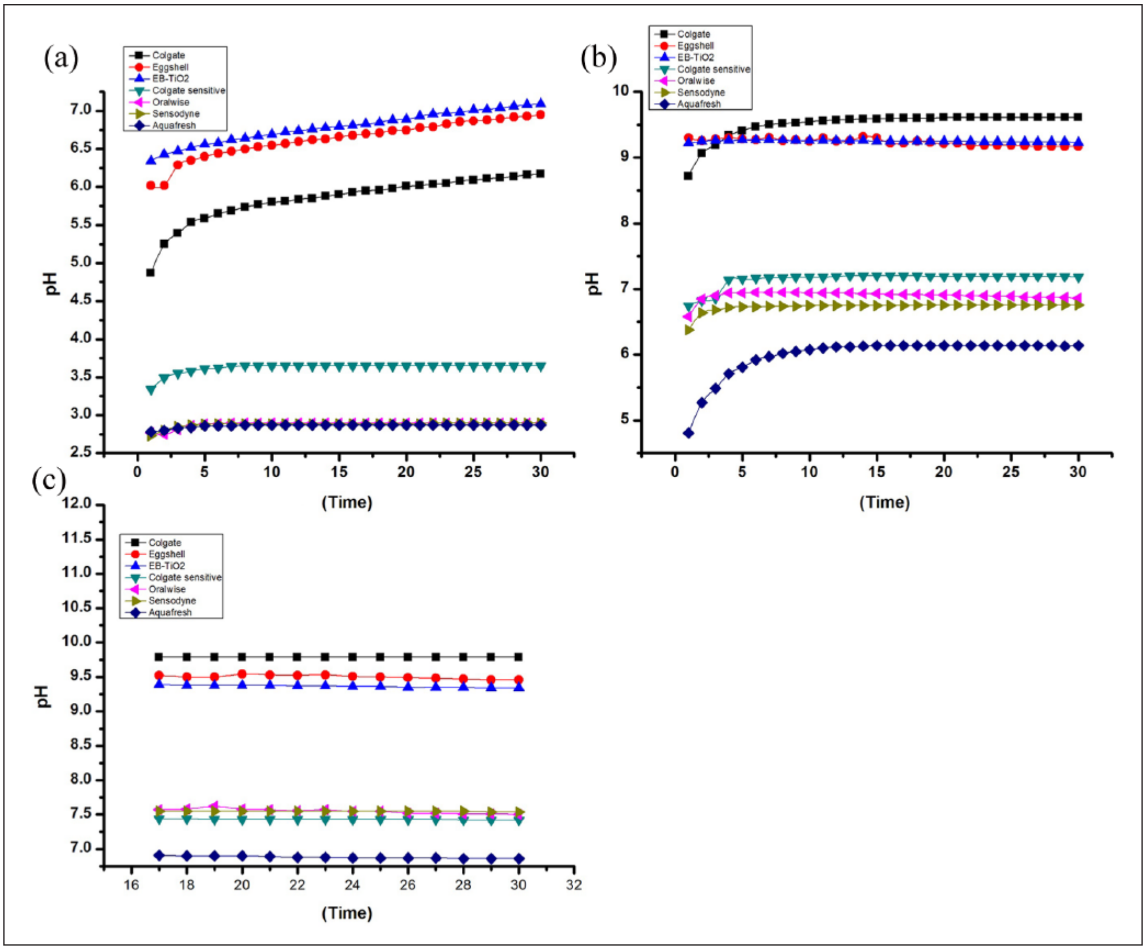

Figure 5. Buffering properties for (a) pH 2, (b) pH 4, and (c) pH 5 after 30 min exposure to citric acid.

properties of eggshell and EB-TiO_2 at each measured pH (2, 4, and 5) were comparable the Colgate brand of toothpaste.

The one-way ANOVA, mean, standard deviation, and standard error results for the resistance of the samples against citric acid exposure are illustrated in Table 1. Significant differences were found in the acid resistance properties of the samples and their exposure to citric acid ($P < 0.05$). The results of the post hoc comparison test are given in Table 1. The k/Pa/s values for the Sensodyne, Colgate, and Aquafresh toothpastes infused with eggshell powder were significantly higher than those for the toothpastes infused with the EB-TiO_2 group ($P < 0.05$). No statistical differences were found between the k/Pa/s values for the Colgate Sensitive and Oralwise groups infused with both eggshell powder and EB-TiO_2 ($P > 0.05$).

Discussion

Calcium carbonate-based material has been extensively studied for its buffering properties, particularly for the treatment of gastro-esophageal reflux disease and peptic ulcers.[41] The calcium carbonate ($CaCO_3$) constituent of EB-TiO_2 (Figure 1(a)) suggests that it can effectively buffer acidic solutions. The buffering activity for the eggshell powder, EB-TiO_2, and the Colgate brand of toothpaste at pH values of 2, 4 and 5, respectively, were much more effective than the other tested commercial toothpastes (Figure 5). The enhanced buffering property of the carbonate-based products is attributed to their higher solubility in acidic solutions. This supports the argument of Raliya et al.[41] that the higher solubility of calcium carbonate-based material results in a greater concentration of ions in solution. Consequently, this leads to a higher buffering activity as the faster dissolution rate increases the pH quickly, thereby expanding the horizon of neutralization.

Equally important, the buffering activities of eggshell powder and EB-TiO_2 were comparable to each other. It can therefore be gathered that the modification of eggshell powder with titanium dioxide does not adversely affect the carbonate structure of EB-TiO_2. From a dental oral health perspective, and corroborating with Macri,[42] the buffering property of EB-TiO_2 is particularly important, as it can increase the pH of an acidic environment where demineralization is likely to occur and offer a high bioavailability of calcium.

Table 1. Analysis of variance (ANOVA) results showing sample resistance against citric acid.

Samples	*N*	Mean	Std. deviation.	ANOVA test (*p*-value)	Post hoc Bonferroni test
Eggshell powder	2	0.0597	0.00788	0.029	1.000
EB-TiO_2	2	0.0465	0.00790		1.000
Sensodyne *(eggshell powder)*	2	0.0316	0.00265		0.024
Sensodyne *(EB-TiO_2)*	2	0.0199	0.00713		1.000
Colgate *(eggshell powder)*	2	0.0616	0.02177		0.005
Colgate *(EB-TiO_2)*	2	0.0608	0.00190		0.364
Colgate Sensitive *(eggshell powder)*	2	0.0401	0.00859		0.498
Colgate Sensitive *(EB-TiO_2)*	2	0.0361	0.00528		1.000
Oralwise *(eggshell powder)*	2	0.0348	0.00036		0.104
Oralwise *(EB-TiO_2)*	2	0.0321	0.00416		0.082
Aquafresh *(eggshell powder)*	2	0.0382	0.02329		0.018
Aquafresh *(EB-TiO_2)*	2	0.0329	0.00299		0.082
Total	24	0.0412	0.01506		

Note: the Colgate brand of toothpaste infused with eggshell powder had the highest mean slope (0.0616 $\pm$ 0.02 kPa/s), while Sensodyne infused with EB-TiO_2 had the lowest mean slope (0.0199 $\pm$ 0.01 kPa/s).

The acid-resistant properties of EB-TiO_2 were evaluated in vitro using a pressure sensor. The mean average slope (kPa/s) was measured and used for statistical analysis. On the basis of the research data, the stated hypothesis was accepted. The experimental results indicate that the addition of EB-TiO_2 to commercial toothpastes statistically affected the acid resistance when exposed to citric acid ($P < 0.05$).

Overall, the kPa/s values measured in the brands of toothpastes added with EB-TiO_2 were consistently below those with eggshell powder alone (Table 1). In light of the differences measured in the acid-resistant properties between EB-TiO_2 and eggshell powder, it can be assumed that this difference is related to the presence of TiO_2. According to García-Valverde et al.,[43] titanium dioxide is reported to have good acid-resistant characteristics. The presence of TiO_2 on the surface of the eggshell powder (Figure 2(b)) confirms that EB-TiO_2 will be effective against acidic attacks. This finding supports Tao et al.[35] in that modification of calcium carbonate-based material with titanium dioxide is more likely to produce a higher resistance to acids. Eggshell powder should therefore be modified with titanium dioxide for reducing erosive attacks.

Conclusions

The prominent feature of this study revealed the successful modification of EB-TiO_2 through ball-milling. The FTIR and XRD results confirmed TiO_2 coating on the surface of eggshell powder. The TEM revealed spherical-shaped particles with a particle size of 13 nm. Conclusively, this paper showed that EB-TiO_2 had good buffering and acid-resistant characteristics. From an oral health perspective, using EB-TiO_2 in toothpaste formulation will provide low cost, affordable, feasible, and sustainable products that are needed to improve the quality of oral health globally.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Research Foundation of South Africa (No. 104824).

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Paper III

Onwubu, S.C., Mdluli, P.S., Singh, S., Madikizela, L. and Ngombane, Y. 2019. Characterization and In Vitro Evaluation of An Acid resistant Nanosized Dental Eggshell-Titanium dioxide Material. *Advanced Powder Technology*, 30 (4): 766-773. https://doi.org/10.1016/j.apt.2019.01.005

Advanced Powder Technology 30 (2019) 766–773

Contents lists available at ScienceDirect

Advanced Powder Technology

journal homepage: www.elsevier.com/locate/apt

Original Research Paper

Characterization and *in vitro* evaluation of an acid resistant nanosized dental eggshell-titanium dioxide material

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ARTICLE INFO

Article history:
Received 10 May 2018
Received in revised form 9 January 2019
Accepted 13 January 2019
Available online 8 February 2019

Keywords:
Eggshell powder
Titanium dioxide
Remineralization
Modification
Acid resistant
Toothpaste

ABSTRACT

This paper reports on novel nanosized acid resistant material base on the modification of eggshell powder and titanium dioxide (TiO_2-EB) for enamel remineralization. The TiO_2-EB was prepared by ball milling eggshell powder and titanium dioxide. Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Scanning Electron Microscope (SEM), Transmission Electron Microscopy (TEM), and ImageJ were used to characterise TiO_2-EB. A computation model using Material Studio Software was used to explain the mechanism of TiO_2-EB interaction. In addition, the acid resistant of TiO_2-EB was evaluated by comparison using three commercial toothpaste. The mean pressure value (kPa/s) was measured using a pressure sensor. The FTIR, XRD analysis confirmed the surface modification of TiO_2-EB. The SEM image revealed that pure TiO_2 particles are spread on the surface of eggshell powder. The TEM image revealed spherical particles in TiO_2-EB. The ImageJ showed the average particle size of TiO_2-EB to be 13 nm. In addition, the commercial toothpastes doped with TiO_2-EB showed an improved acid resistant. The salient features of this study indicate that TiO_2-EB will effectively remineralized enamel lesions while offering better protective covering to the enamel.

1. Introduction

Eggshells are material mainly produced by poultry and domestic kitchens waste. In recent years, the use of liquid eggs at industrial level has resulted in a considerable amount of eggshells waste as a by-product [1]. Most of the residual waste eggshells from these industries are mainly discarded into landfills without further treatment, and subsequently overburdening the environment [2,3]. In the United States, for example, 150,000 tons of eggshells waste is disposed in landfills annually which create land availability problem [4]. More so, and from economic perspective, Das, Minkara, Melear and Tollner [5] and Chai [6] pointed out that industries incur high costs in the disposal of eggshells. Given the amount of eggshells waste that needs to be disposed worldwide, it is important to find alternative mea ns of converting eggshells waste materials into value added products for environment sustainability.

Eggshell is a natural bioceramic composite that has a unique chemical make–up of inorganic and organic compounds. It consists of an inorganic shell and an organic membrane. The shell together with its membrane weighs approximately 11% of the egg weight. The inorganic constituent of eggshells is mainly calcium carbonates (∼95% in the form of calcite), whereas it's organic compounds are a matrix of proteins, glycoprotein, proteoglycan, and type X collagen sulphated polysaccharides (∼5%) [7–9]. Owing to this unique chemical composition, eggshell has extensively gained attention among researchers for its medical and dental benefits. For example, Khandelwal and Prakash [10] demonstrated how calcium carbonate derived from eggshells could be used to produce hydroxyapatite, which is mainly used in bone and dental treatments. Abdulrahman, Tijani, Mohammed, Yusuf, Jibrin and Mohammed [11] alleged that eggshells based hydroxyapatite and nanohydroxyapatite could reduce the cost of treatment in bone repair or replacement. Murakami, Rodrigues, Campos and Silva [12] noted that calcium carbonate from eggshells has great potential in the development of alternative ingredients for medical

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https://doi.org/10.1016/j.apt.2019.01.005
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applications such as food additive and calcium supplement, and as a component for bone implants.

Furthermore, eggshells are currently being investigated for their remineralization capabilities [13]. More recently, Mony, Ebenezar, Ghani and Narayanan [14] and Haghgoo, Mehran, Ahmadvand and Ahmadvand [15] reported that eggshell has a rich bioavailable calcium content, which favours the remineralizing of caries lesions. This is particularly important, as enamel remineralization is effective in the prevention of tooth decay process [16,17]. Of Interest, Arnold, Prange and Naumova [18] reveal that calcium carbonate constituent of eggshell are soluble in acidic environment; which become unstable upon dietary challenge. This is concerning, as acidic oral environment have been reported to demineralized the enamel thereby provoking tooth decay [17,19–21]. Modifying eggshell is therefore critical if is to be used in the oral environment for enamel remineralization. Several studies have indicated that nanosized titanium dioxide can improve the acid resistant properties of calcium carbonates [22,23]. In particular, mechanical milling of the titanium dioxide and calcium carbonate effectively breaks and modifies the surfaces of $CaCO_3$ nanoparticles which greatly improved its acidic properties [22]. While the use of TiO_2 in the modification of $CaCO_3$ nanoparticles by mechanical milling have been extensively studied in papermaking industry [22–24], there is, however, limited research on its use in the modification of eggshell powder for dental application. This paper therefore aimed to characterize and evaluate the acid resistant properties of a novel nanosized eggshell-titanium dioxide material (TiO_2-EB) in order to determine its suitability for enamel remineralization. The formulated hypothesis was that modifying eggshell with titanium dioxide will improved its acid resistant characteristics.

2. Materials and methods

All chemicals used in this study were of analytical grade. Food grade anatase titanium dioxide (CAS No: 13463677) was purchase from Sigma-Aldrich (Germany). Citric acid Monohydrate - was supplied by Merck (South Africa). Three different brands of toothpastes were bought from a popular shopping mall located at Durban (South Africa). These toothpastes included: Sensodyne, Colgate sensitive toothpaste, and Colgate. The composition, active ingredients and manufacturers of the toothpaste are given in Table 1.

2.1. Preparation of nanosized dental eggshell-titanium dioxide composite (TiO_2-EB)

Eggshells collected from food outlets were washed with regular detergent to remove impurities. Eggshells were then disinfected by storing the eggshells in a diluted solution of household sodium hypochlorite for six hours. Subsequently, eggshells were vacuum dried for ±6–9 min at 250 °C. Modification of eggshell with titanium dioxide was achieved in two steps. In the first step, eggshells were ball-milled by placing 30 g of the eggshell in a 500 mL stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10 mm diameter and dry-milled in a planetary ball mill (Retsch ® PM 100) at 400 rpm for 20 min. The collected powder was sieved to a particle size of ≤25 μm using a mechanical sieving shaker (Retsch AS 200, Germany). The eggshell powder and titanium dioxide mixing ratio was optimized following the procedure reported by Lin, Dong and Jiang [22], 20 g of the fine eggshell powder obtained in step 1 were modified by adding 5 g of food grade anatase titanium dioxide (≤15 μm. The mixture was subsequently ball-milled for 200 min to obtain a nanosized eggshell-titanium dioxide material (Fig. 1).

2.2. Characterization of TiO_2-EB

2.2.1. Fourier transform infrared spectroscopy analysis

The infrared spectra were measured using a Perkin Elmer Universal ATR spectrometer to identify the functional group constituents of TiO_2-EB.

2.2.2. X-ray diffraction analysis

The X-ray diffraction (XRD) analysis was performed to observe the possible changes in crystallinity between the eggshell powder, titanium dioxide, and TiO_2-EB. The XRD patterns were recorded using a diffractometer (PANalytical-Empyrean instrument; Co radiation 1.54056 Å) and analysed between 0 and 90° (2 theta). The voltage, current and pass time used were 40 Kv, 40 mA and 1 s, respectively.

2.2.3. Energy dispersive X-ray spectroscopy and scanning electron microscope analysis

Energy dispersive x-ray spectroscopy was used in conjunction with scanning electron microscope (Field Emission-Carl Zeiss) operating at controlled atmospheric conditions at 20 kV to examine the surface morphology and the elemental composition of the TiO_2-EB. As a proxy measurement, pre and post agitation of TiO_2-EB after 30 min with ultrasound technology (UP4DOS, Hielscher) was carried out to establish the strength of modification. Prior to SEM observation, the surface was coated with a thin, electric conductive gold film to prevent build-up of electrostatic charge.

2.2.4. Transmission electron microscopic analysis

A Transmission Electron Microscope (TEM) was used to observe the particle size, shape and distribution of TiO_2-EB. Very small quantities of TiO_2-EB were dispersed in 10 mL ethanol and sonicated at 10 kv for 10 min. Subsequently, thin cross-sections of cryo-microtomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids. Anal-

Table 1
Brand, composition, and manufacturer of toothpastes.

Toothpaste	Composition as indicated in toothpaste labels by supplier	Manufacturer
Colgate	Sodium monofluorophosphates, Calcium carbonates, Aqua, Sorbitol, Sodium lauryl sulfate, Aroma, Cellulose gum, Sodium bicarbonate, Tetrasodium pyrophosphate, Benzyl alcohol, Sodium saccharine, Sodium hydroxide, limonene	Colgate-Palmolive Co.
Colgate sensitive	Aqua, Gycerin, Hydrated silica, Sorbitol, Potassium Nitrate, PEG-12, Tetrapotassium pyrophosphate, Sodium lauryl sulfate, Zinc citrate, PVM/MA copolymer, Aroma, Potassium hydroxide, Xanthan gum, Cellulose gum, Cocamidopropyl Betaine, Sodium Fluoride, Sodium Saccharin, Eugenol, and Limonene	Colgate-Palmolive Co.
Sensodyne	Aqua, Sorbitol, Hydrated silica, Glycerin, Potassium Nitrate, Cocamidopropyl Betaine, Aroma, Xanthan gum, Titanium dioxide, Sodium Fluoride, Sodium saccharin, Sodium hydroxide, Sucralose, and Limonene	GlaxoSmithKline

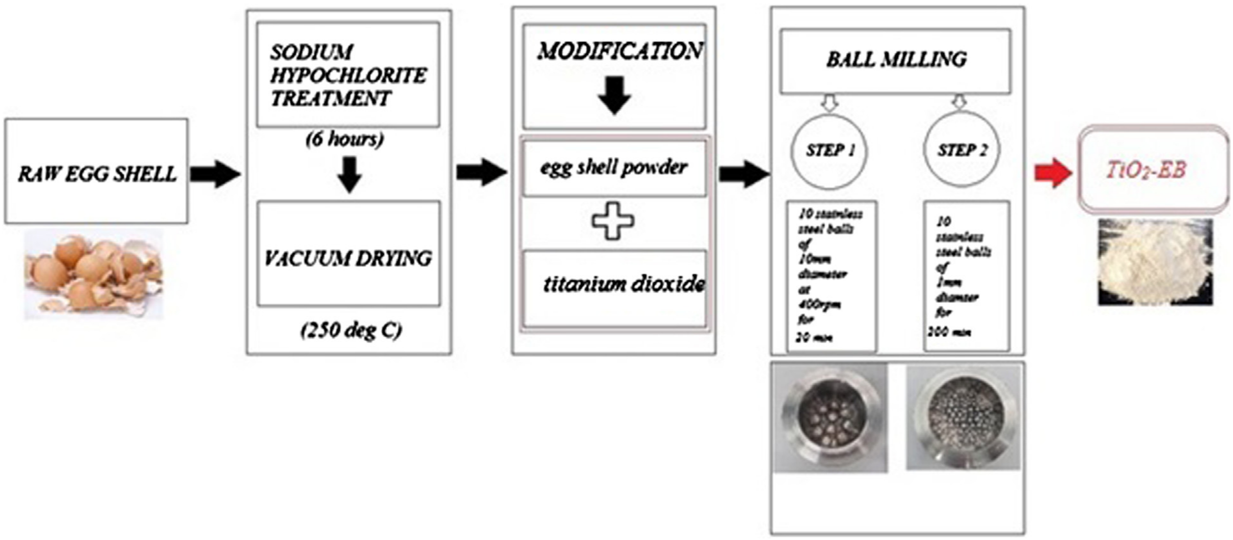

Fig. 1. Schematic illustration of different steps in the preparation of TiO_2-EB.

ysis was conducted using a transmission electron microscope (TEM-Philips CM 120 model) at 120 kV.

2.2.5. Computation of TiO_2-EB structural interaction using material studio and EDX mapping

Material studio software (version 6) was used to analyze the structural interaction of the calcite and titanium dioxide component of TiO_2-EB. EDX mapping was further used to validate the computation interaction.

2.3. Evaluation of acid resistant properties for TiO_2-EB

The acid resistant characteristics of TiO_2-EB were evaluated by comparison using simulated eggshell enamel model. This entails doping commercial toothpastes with TiO_2-EB and eggshell powder, respectively. It was assumed that if TiO_2-EB has improved acid resistant properties then it would be a suitable material for the remineralization of tooth enamel.

2.3.1. Preparation of simulated eggshell enamel samples

Eggshell enamel samples were simulated *in vitro* using TiO_2-EB and eggshell powder (control). 3 g of each brand of toothpaste listed in Table 1 were dissolved in a beaker containing 100 cm^3 deionized water by constant agitation using magnetic stirrer at 800 rpm for 20 min. 1 g of the prepared eggshell powders and TiO_2-EB was added to the beaker containing the dissolved toothpastes, respectively. These were further agitated for 8 h at a speed of 800 rpm. The mixtures were then filtered and subsequently oven dry at 60° for 3 h. The prepared samples were then exposed to 2 mol L^{-1} hydrochloric acid (HCl).

2.3.2. pH test

1.5 g of each samples were placed in a beaker containing 50 mL deionized. The solution were constantly agitated at low speed of 600 rpm for 30 min. A pH meter (Starter 300, Ohaus Incorporation USA) equipped with temperature sensor was used to record changes in the pH reading.

2.3.3. Pressure sensor test

A gas pressure sensor (Vernier LabPro, USA) was used to monitor pressure changes in the pressure (kPa) against time (s) during the reaction of the samples with HCl. Erlenmeyer flask (250 mL) was used as the reaction container during the pressure test. A stopper was inserted into the flask to provide an airtight vessel. This was then connected to plastic tubing attached to a gas pressure sensor (Order Code GPS-BTA). With the aid of an interface system (Vernier LabPro) attached to a computer, the pressure readings were collected and analysed using LoggerPro 3 software. Prior to the experiment, 0.5 g of each sample was placed in the Erlenmeyer flask, while 25 mL of the prepared HCl was used as the acid reactant. All tests were performed in duplicate, and the mean slope (kPa/s) values were used for the statistical analysis.

2.4. Evaluation of acid resistant properties for TiO_2-EB in situ

Fourteen freshly collected bovine enamel were used to evaluate the acid resistant properties of TiO_2-EB in situ. The tooth samples were sectioned to a diameter of 5 mm × 5 mm × 3 mm using a diamond cutter under water to avoid overheating. The samples were then randomly assigned to 7 groups of 2 specimens each. 1: Unexposed enamel; 2: Exposed enamel 3: Exposed enamel in Colgate solution; 4: Exposed enamel in Sensodyne solution; 5: Exposed enamel in Colgate sensitive solution; 6: Exposed enamel in EB-TiO_2 solution, and 7. Exposed enamel in eggshell alone.

3. Results and discussion

3.1. Characterization of TiO_2-EB

Following the successful modification of eggshell powder and titanium dioxide through ball-milling, the FT-IR spectra of eggshell powder, titanium dioxide, and TiO_2-EB in Fig. 2 revealed several bands from 500 cm^{-1} to 2000 cm^{-1}. The band aspect of the FT-IR spectra shows difference between the eggshell powder (Fig. 2A), titanium dioxide (Fig. 2B), and TiO_2-EB (Fig. 2C). As shown in Fig. 2A, there are prominent absorption peaks of carbonates at 1411 cm^{-1}, which is associated to the carbonyl group found in calcium carbonate. Furthermore, the FT-IR spectra shows the absorption peak of calcite around 711 and 873 cm^{-1}. These were attributed to asymmetric and symmetric stretching, out-of-plane bending and in-plane bending vibration modes for calcium carbonate (CO_3^{2-}) molecules [8,25–27]. In contrast to eggshell powder in Fig. 2A, TiO_2-EB in Fig. 2C shows a broad, intense band below 1000 cm^{-1}. This corresponds to the Ti-o-Ti vibrations of the titanium dioxide [23,28].

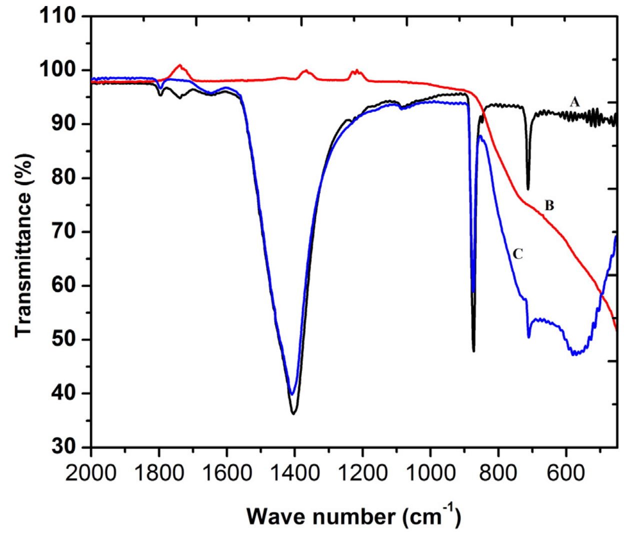

Fig. 2. FTIR spectra showing (A) eggshell powder; (B) TiO_2-EB (C) titanium dioxide.

From Fig. 3, the XRD pattern of eggshell powder, titanium dioxide and TiO_2-EB showed characteristic diffraction peaks with diffraction angles at 29.5°, 34.5°, 42.5°, 46.5°, 50.5°, 56°, 57°, 67°, 68° and 72°. For Fig. 3A, the characteristic peak marked around 34.5° (2θ) indicates the presence of thermodynamically stable calcite crystalline structure, which is similar to calcium carbonate [8]. As shown in Fig. 3B, the XRD pattern of pure TiO_2 shows a characteristic diffraction peak with values lying at 2θ = 29.5° corresponds to anatase phase and is confirmed with International Centre for Diffraction Data (ICDD Ref: 98-009-6946). The TiO_2-EB diffraction peak shown in Fig. 3C revealed the presence of calcite and anatase crystalline structure. Equally important, and consistent with Tao, He and Zhao [23], the shape, intensity, and location of the TiO_2-EB peaks corresponding to the pure titanium dioxide, is an indicative of the deposition of TiO_2 on the surface of the $CaCO_3$.

The results given in Table 2 illustrates the elemental composition of eggshell, titanium dioxide, and TiO_2-EB (pre and post agitation). Specifically, it can be observed that the elemental composition of TiO_2-EB pre-and post-agitation were almost similar. Thus suggesting that the bond strength between titanium dioxide and eggshell powder was highly effective.

The SEM images of eggshell powder, pure anatase titanium dioxide, and TiO_2-EB composite particles (pre and post) agitation are given in Fig. 4. As seen in Fig. 4A, the SEM images of eggshell powder shows more irregular shaped particles. It can be observed

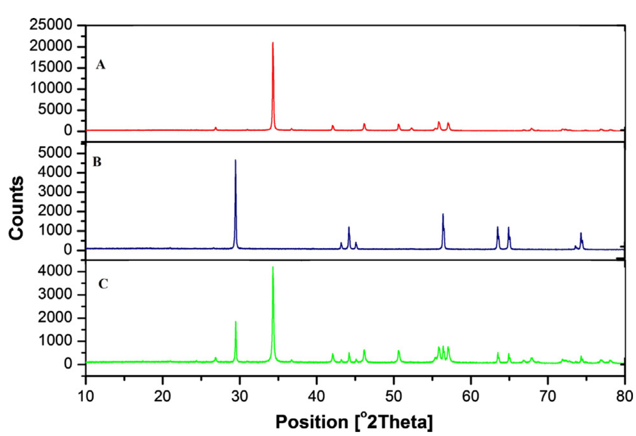

Fig. 3. XRD pattern of (A) eggshell powder; (B) titanium dioxide; (C) TiO_2-EB.

that approximately cubic and spherical particles coexisted (Fig. 4A). For the image shown in Fig. 4B, nearly spherical like morphological can be seen for TiO_2. For Fig. 4C, it can be observed that the pure anatase particles is scattered on the surface of TiO_2-EB. More so, it can be seen that titanium dioxide sufficiently coated the eggshell powder particles and formed a compact layer of TiO_2 firm. For Fig. 4D, similar evenly spread of TiO_2 on the surface of the eggshell powder particles was observed post agitation, thereby confirming the bond strength between eggshell powder and titanium dioxide.

The particle size and shape of TiO_2-EB were characterized by TEM. As shown in Fig. 5, the presence of nonhomogeneous structure with agglomeration of particles can be observed. Equally, a TEM analysis revealed distribution of spherical shape particles of different sizes corroborating with the SEM analysis. In addition, the particle size distribution of TiO_2-EB were further analysed using ImageJ software (National Institute of Health USA, http://imagej.nih.gov./ij). As seen in Fig. 5B, the average mean particle size of TiO_2-EB was revealed to be around 13 nm.

A computation model showing the reaction between calcite structure and anatase is proposed in Fig. 6. As shown in Fig. 6C, the TiO_2 formed a reaction bond with the calcite structure of the eggshell at the surface, which helps to explain the mechanism of coating. As suggested in literature [22], large proportions of CO_3^{2-} on the surface of $CaCO_3$ particles react with moisture during the mechano-chemical process to produce $CaCO_3$ hydroxyl compounds. More so, these hydroxyl compounds are the active point that facilitate modification [29]. In addition, and consistent with Tao, He and Zhao [23] $CaCO_3$ and TiO_2 molecules reaction on the surface is depended on Vander Waals forces to hold them together. These forces play an important role in the intermolecular interactions between calcite structure and anatase. Fig. 6D shows the mapping generated from the EDX elemental analysis. The evenly spread of TiO_2 on the surface of eggshell powder particles strongly suggests the surface modification of TiO_2-EB.

3.2. Evaluation of the acid resistant properties of TiO_2-EB

The mean pH of tested toothpastes, eggshell, and TiO_2-EB is given in Fig. 7. The pH results suggests that calcium carbonates base materials such as Colgate (9.61) eggshell (9.37), TiO_2-EB (9.31) have strong alkaline characteristics. On the contrary, the toothpastes brands such as Colgate Sensitive (7.4), and Sensodyne (7.41) had a pH value that can be considered neutral.

Fig. 8 illustrates the mean pressure slope value of the eggshell powder and TiO_2-EB that are doped into three commercial toothpastes, namely: Colgate, Sensodyne, and Colgate Sensitive. In contrast to eggshell powder, toothpaste samples doped with TiO_2-EB showed improved acid resistant properties.

Fig. 9 demonstrate the acid resistant properties of the tested commercial toothpastes, eggshell and TiO_2-EB. Fig. 9(B), (D), and (E) visibly showed the demineralization of the tooth samples against acidic challenge in the presence of the tested toothpastes. Equally, the enamel surface were observed to be rougher and showing more destruction of the tooth prismatic structure. On the contrary, enamel samples exposed to acid in the presence of eggshell and Colgate showed rougher surface but less destruction of prismatic structure of the tooth (Fig. 9C and F). The less prismatic destruction observed for these samples (C & F) could be attributed to the high alkaline properties of the materials. In contrast, the image in Fig. 9G suggests that the acid had no effect on the enamel in the presence of TiO_2-EB, as similar images were observed with that of the unexposed enamel (Fig. 9A). The image observed in Fig. 9G visibly confirmed that modification of eggshell with titanium improves its resistant in acidic environment.

Table 2
EDX analysis showing elemental composition.

Element	Eggshell powder	Titanium dioxide	TiO_2-EB	
			Pre agitation	Post agitation
Calcium (Ca)	22.66	0.00	14	15.24
Oxygen (O)	55	48.14	52.39	51.86
Titanium (Ti)	0.00	51.58	12.31	17.49
Magnesium (Mg)	0.37	0.00	0.2	0.18
Carbon (C)	21.98	0.00	21.09	15.23

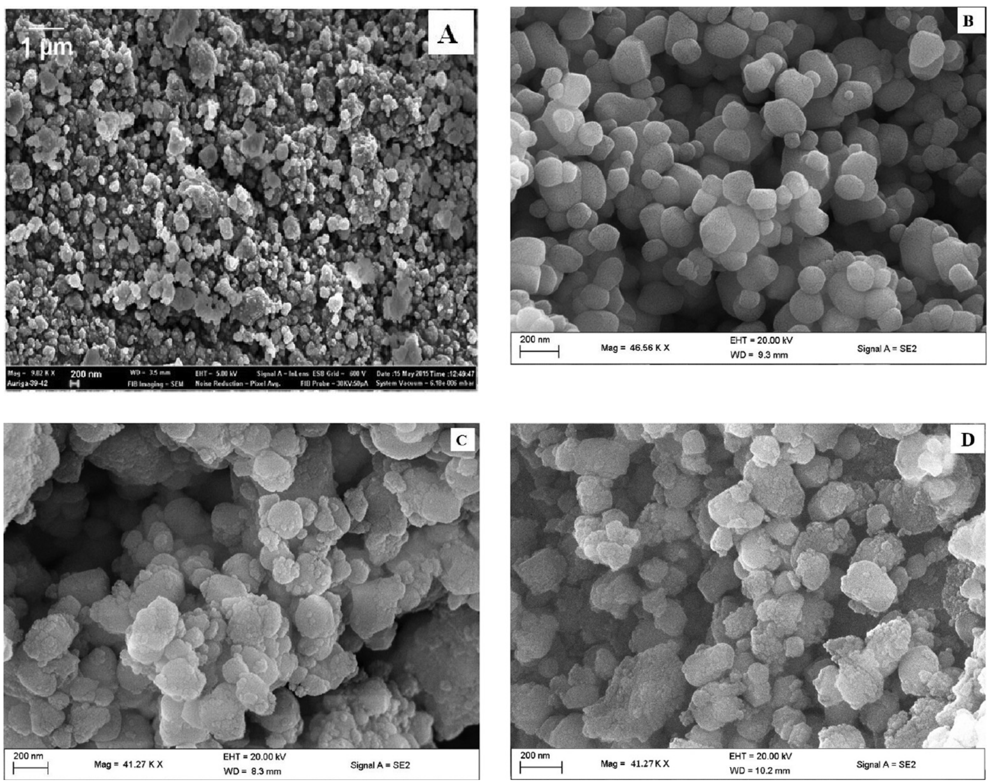

Fig. 4. SEM micrograph for (A) eggshell powder; (B) titanium dioxide; (C) TiO_2-EB before agitation; (D) TiO_2-EB after agitation.

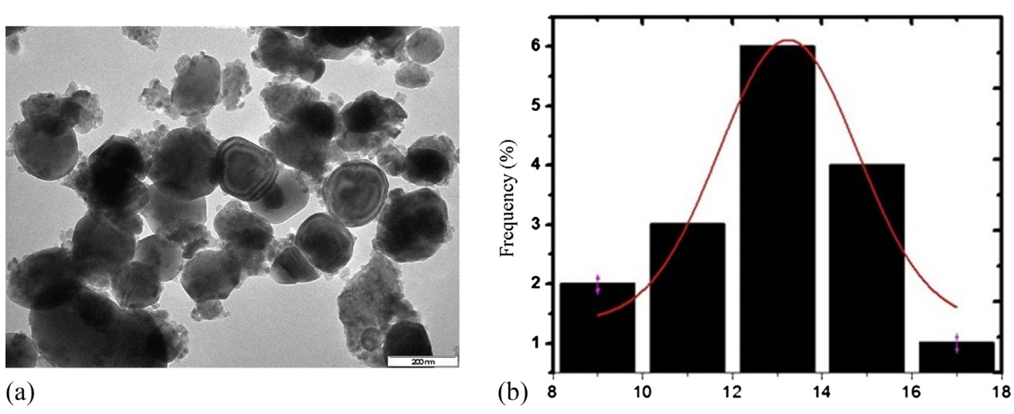

Fig. 5. TEM images: (A) Images of TiO_2-EB after ball-milling for 200 min; (B) Normal distribution of TiO_2-EB particle size.

3.3. Discussion

Eggshell powder have been predicted to be the future of enamel remineralization. The findings from this study suggests that the poor acid resistant of calcium carbonate constituent of eggshell powder may limit its use in a high acidic environment. A critical point deserving mentioning is that high acidic environment help facilitate enamel demineralization (Fig. 9B). The presence of titanium dioxide coating on the surface of TiO_2-EB was highly effective in the prevention of acidic attack (Fig. 9G). Eggshell powder should

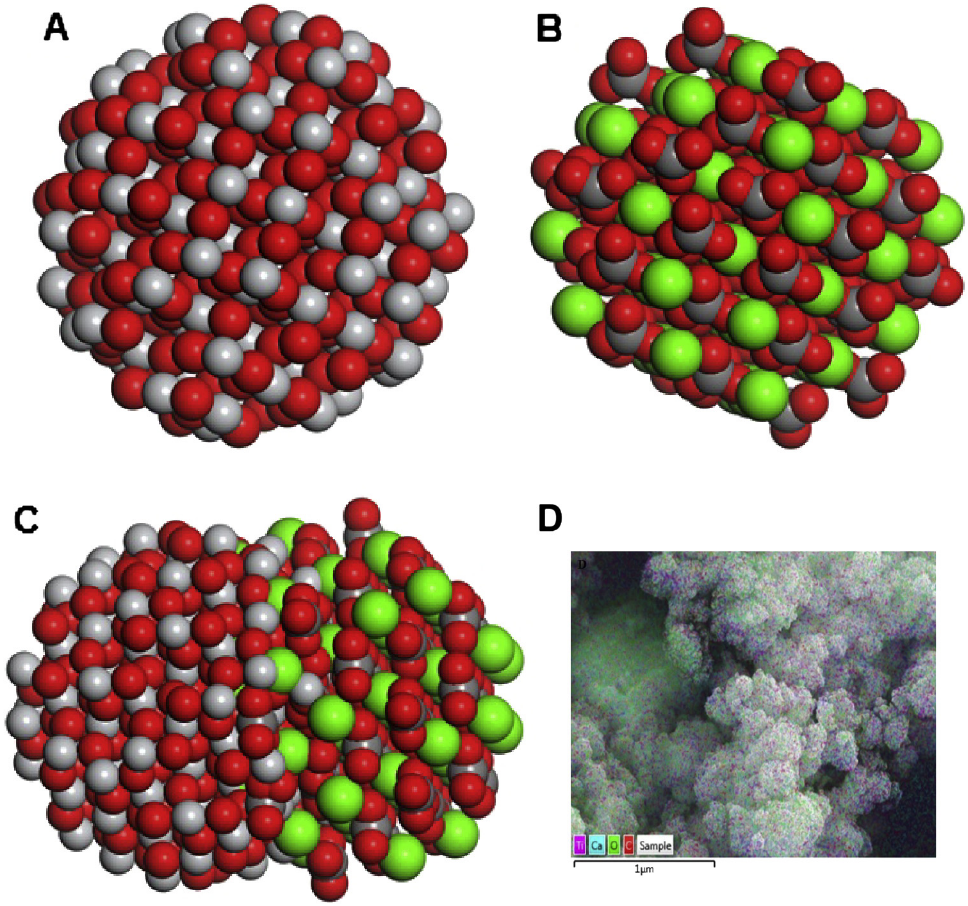

Fig. 6. (A) molecular structure for titanium dioxide; (B) molecular structure of calcite (C) structural interaction of calcite-titanium dioxide; (D) EDX mapping of TiO_2-EB.

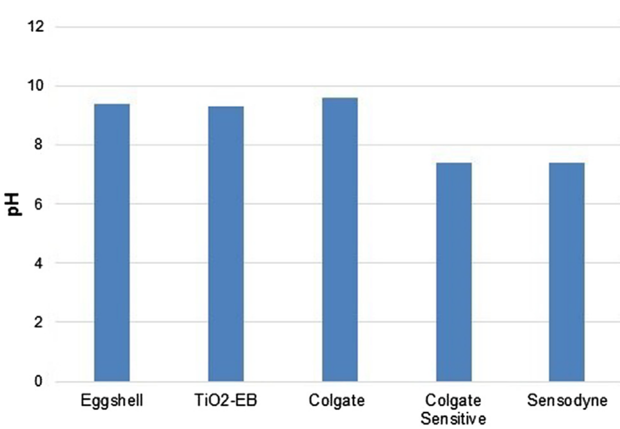

Fig. 7. pH of commercial toothpastes, eggshell and TiO_2-EB.

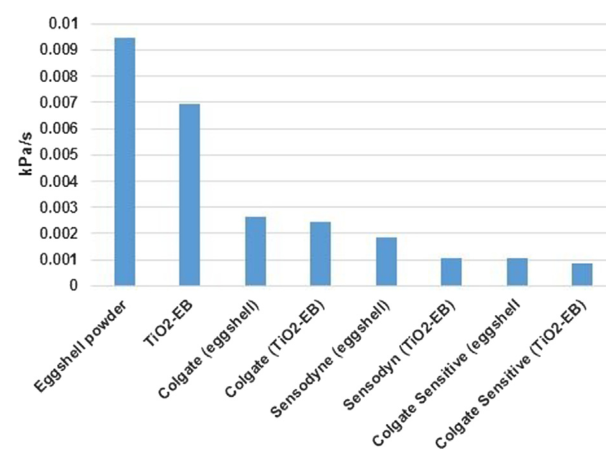

Fig. 8. Mean slope differences between eggshell and TiO_2-EB showing acid resistant properties TiO_2-EB.

therefore be modified with titanium dioxide when used in toothpaste for enamel remineralization.

Moreover, the in-plane bending vibration modes of calcium carbonates molecules (Fig. 2c) as well as the diffraction peak of calcite (Fig. 3C) strongly suggests that modification of eggshell powder with titanium dioxide does not adversely affect the carbonate structure of TiO_2-EB. This is particularly important as it can increase the pH of an acidic environment (Fig. 7) where demineralization is likely to occur and offer a high bioavailability of calcium [13]. Equally important, the improved acid resistant properties measured in the toothpastes doped with TiO_2-EB further supports titanium dioxide forming a coating on the surface of the eggshell powder. This finding is consistent with [30]. Consequently, the hypothesis was accepted as modification of eggshell powder with titanium dioxide showed an improved acid resistant. Thus suggesting TiO_2-EB suitability as alternative to eggshell for enamel remineralization.

From a dental material perspective, and corroborating with Rahardjo, Nugraheni, Humaira, Adiatman and Maharani [31], the particle size of TiO_2-EB (Fig. 5B) is more likely to effectively remineralized enamel lesions. Furthermore, Cutler [32] suggested

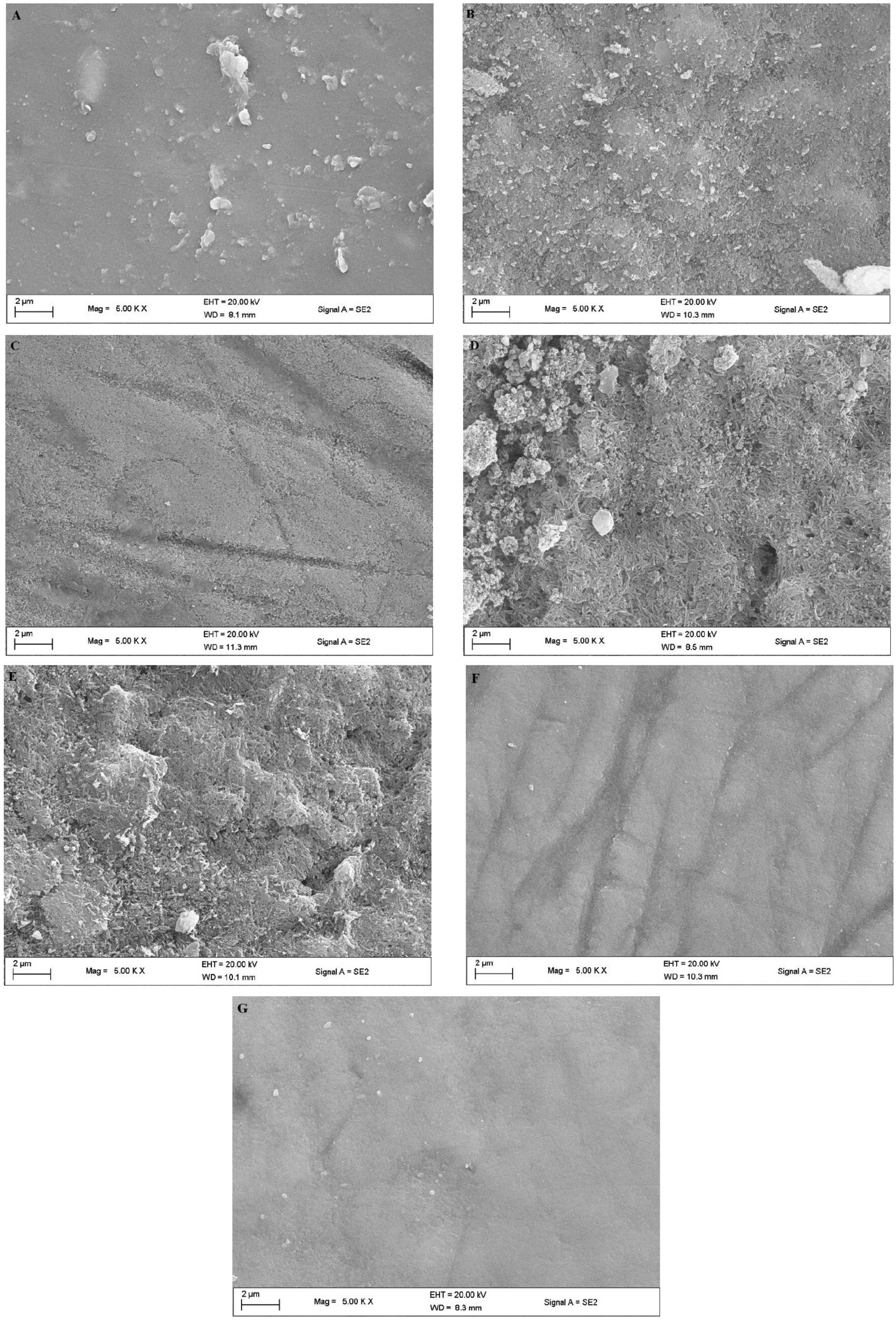

Fig. 9. Enamel surface (A) Unexposed tooth; (B) after exposure to HCl; (C) after exposure to Colgate toothpaste; (D) after exposure to Colgate Sensitive; (E) after exposure to Sensodyne (F) after exposure to eggshell; (G) after exposure to TiO_2-EB (All exposed samples were in a solution of 0.2 mL HCL for 30 min).

that nanosized titanium dioxide and dental abrasive agents can be used together in the remineralization of damaged teeth. In light of these findings, it is expected that TiO_2-EB will effectively remineralized enamel lesions, at the same time, offering better protective covering to the enamel against erosive challenged than eggshell alone.

In addition to these, anatase TiO_2 have been reported to be an effective antimicrobial agents that is compatible with human body or environment [33,34]. In particular, and in a more recent study, it was demonstrated that titania reduces that ability of bacterial such as *Staphylococcus aureus* (*S. aureus*) to adhere to surfaces by rupturing their cell membrane [35]. Given that the metabolic activities of bacterial in the oral cavity contribute to enamel demineralization and subsequently dental caries [36], this study assumed that due to the presence of titania (TiO_2), TiO_2-EB could also act as antimicrobial agents in the prevention of dental caries. This is an area worth exploring in the future.

4. Conclusion

The prominent findings of this paper showed that titanium dioxide effectively coat the surface of eggshell powder. The functional groups, surface morphology, particle size and mechanisms of coating were confirmed by suitable characterization techniques. Notably, this study conclusively showed that TiO_2-EB will effectively remineralized enamel lesions while offering better protective covering to the enamel against acid attack. This is an area for further research.

Acknowledgment

The authors acknowledged the financial support from the National Research Foundation of South Africa (No. 104824).

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Paper IV

Onwubu, S.C., Mdluli, P.S., Singh, S., Nyembe, S. and Thakur, R 2018. An In situ evaluation of the protective effect of nano-eggshell/titanium dioxide against erosive acids. *International Journal of Dentistry*, 2018:1-8.
https://doi.org/10.1155/2018/4216415

Hindawi
International Journal of Dentistry
Volume 2018, Article ID 4216415, 8 pages
https://doi.org/10.1155/2018/4216415

Research Article

An In Situ Evaluation of the Protective Effect of Nano Eggshell/Titanium Dioxide against Erosive Acids

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Received 18 August 2018; Revised 19 October 2018; Accepted 7 November 2018; Published 2 December 2018

Academic Editor: Louis M. Lin

Objectives. Enamel erosion caused by high consumption of acidic drinks poses a significant public health concern. This study was aimed to determine the protective effect of eggshell-titanium dioxide composite (EB@TiO_2) against erosive acids on tooth enamel. *Methods*. Twenty prepared bovine tooth enamel specimens were randomly assigned to 5 sample groups ($n = 4$): (1) unexposed tooth enamel; (2) exposed tooth enamel + HCI; (3) exposed tooth enamel + HCI + Colgate toothpaste; (4) exposed tooth enamel + HCI + Sensodyne toothpaste; and (5) exposed tooth enamel + HCI + EB@TiO_2. The mean roughness value (R_{rms}) of the exposed and unexposed tooth was measured with atomic force microscope (AFM). Scanning electron microscope (SEM) and Raman spectroscopy techniques were used to evaluate the surface morphology and changes. ANOVA was used to analyze the mean square roughness (R_{rms}) values for all specimens. Bonferonni correction was used to identify the mean differences among the 5 groups ($\alpha = 0.05$). The R_{rms} values measured for the unexposed and exposed specimens in HCI alone were statistically significant ($P < 0.05$). *Results*. No significant differences were found for the unexposed and exposed specimens in HCI + toothpaste and EB@TiO_2. The tooth enamel specimens exposed to HCI + Sensodyne had the highest R_{rms} values, while specimens exposed to HCI + EB@TiO_2 had the lowest R_{rms} values. *Conclusions*. This study confirms that the investigated toothpaste provides protection against acidic substances. The study results further suggests that EB@TiO_2 could be used to provide enhanced protection for tooth enamel.

1. Introduction

Enamel erosion has become a topical issue in recent years among clinicians and oral health-care providers due to the increase in the consumption of acidic drinks such as soft drinks, energy drinks, and fruit drinks [1]. Previous studies [2, 3] reported that these drinks have a pH [2–4] value that facilitates the dissolution of the tooth and the destruction of the mineral composition of the enamel. Several other authors [4, 5] have demonstrated that as a result of consumption of acidic drinks, the onset of caries and enamel erosion correlate positively together.

Whilst the tooth enamel is mainly composed of hydroxyapatite in the form of phosphate ions (PO_4^{3-}) and calcium ions (Ca^{2+}), Shellis et al. [6], and Lussi and Carvalho [7] argued that there exists an equilibrium between the tooth enamel crystals and the surrounding oral environment. The destabilization of these equilibrium, particularly when the oral environment pH drops below a critical level (5.5 for enamel and 6.2 for dentin), may result in the dissolution of tooth mineral composition [8]. Moreover, the loss of enamel material by erosion is a dynamic process occasioned with periods of demineralization and remineralization (Figure 1); hence, preventive measures against enamel dissolution from

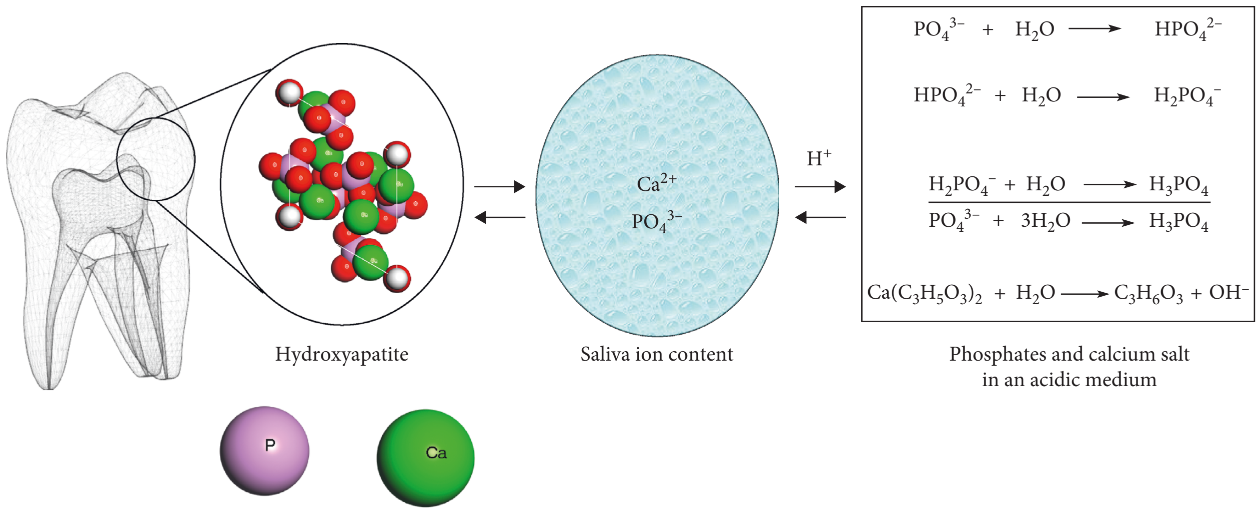


Figure 1: Illustration of tooth demineralization and remineralization dynamism in oral and acidic environment.

acid and potentially permanent damage should be a priority for oral health-care providers [2, 3].

Traditionally, toothpastes have been considered effective and accessible vehicles to improve enamel resistant against erosive oral environment [9]. In recent years, different ingredients have been added to the toothpastes composition to enhance their protective effect against erosive substances. Amongst these ingredients, the use of topical fluoride to modify the effects of erosion at the tooth surface is well documented [10–13]. However, Moron et al. [14] stressed that conventional fluoride-containing toothpastes lack the capacity to protect sufficiently well against erosive substance. Furthermore, Larsen and Richards [15] revealed that at pH below 3, the protective effect of fluoride is diminished. Consequently, it has been suggested that the beneficial health effects against erosive attacks can be improved with the addition of calcium or calcium containing material into toothpaste [16].

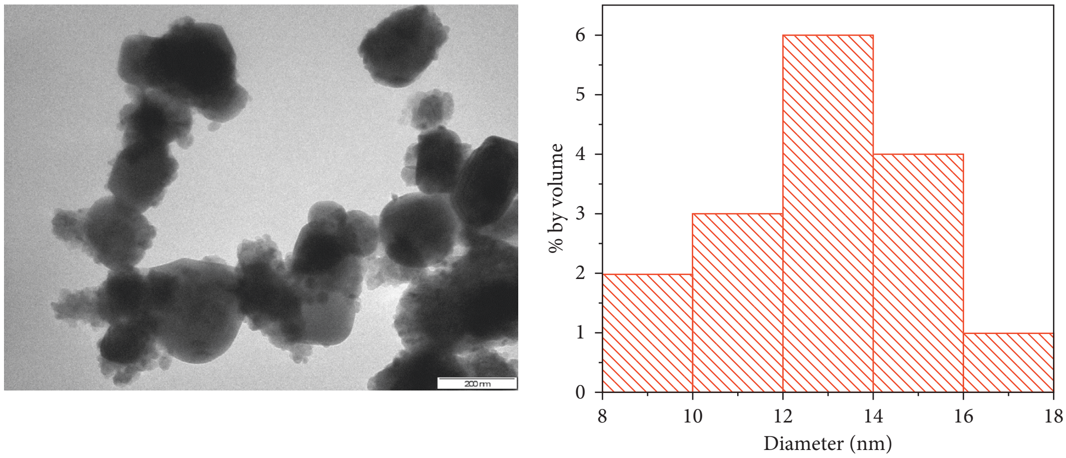
Significantly, studies by King'Ori [17] and Onwubu et al. [18] suggest that the calcium of eggshell can be used as an abrasive cleaner for dental plaque in toothpaste. Lin et al. [19] and Tao et al. [20] equally demonstrated that the mechanical modification of calcium carbonates together with titanium dioxide improved the acid resistance properties when used in paper making industry. However, there is a limited research on the protective effect of a modified eggshell-titanium dioxide composite (EB@TiO_2) on tooth enamel. This in vitro study aimed to determine the protective effect of a modified eggshell-titanium dioxide composite EB@TiO_2 against erosive acids. The protective level of EB@TiO_2 against erosive acids was compared against Colgate® and Sensodyne® toothpaste. It was hypothesized that EB@TiO_2 will offer more protection against erosive challenge than the commercial toothpastes.

2. Materials and Methods

2.1. Preparation of Eggshell-Titanium Dioxide Composite (EB@TiO_2). Modification of eggshell with titanium dioxide was achieved in two steps. In the first step, eggshells were prepared according to the procedure described by Onwubu et al. [21]. Eggshells were disinfected by storing them in a diluted solution of household sodium hypochlorite for six hours. Subsequently, eggshells were vacuum-dried for ± 6–9 min at 250°C. Thereafter, 30 g of the dried eggshell was placed in a 500 ml stainless jar (inner diameter of 100 mm), together with 50 stainless steel balls of 10 mm diameter, and dry-milled in a planetary ball mill (Retsch® PM 100) at 400 rpm for 20 minutes. The collected powder was sieved to a particle size of ≤ 25 μm using a mechanical sieving shaker (Retsch AS 200, Germany). In the next step, 20 g of the fine eggshell powder obtained in step 1 was modified by adding 5 g of anatase titanium dioxide (≤15 μm). The mixture was subsequently ball-milled for 200 min to obtain eggshell-titanium dioxide composite (EB@TiO_2). The particle size distribution of the composite measured using high-resolution transmission microscope (TEM-Philips CM 120 model) at 120 kV and image J software (National Institute of Health USA, http://imagej.nih.gov./ij) was found to be 14 nm (Figure 2).

2.2. pH Measurement. The pH and buffering characteristics of the EB@TiO_2 (tested samples) and the respective toothpastes, brand names, and manufacturers are given in Table 1. The pH of each of the samples in deionized water as well as in 0.01 mol·L^{-1} hydrochloric acid (HCI) were measured by placing 1.5 g of each samples in a beaker containing 50 mL deionized and HCI, respectively. The solution was constantly agitated at low speed of 600 rpm for 30 min. A pH meter (Starter 300, Ohaus Incorporation USA) equipped with temperature sensor was used to record changes in the pH reading. Before testing the samples, the pH was calibrated using tested buffer of known pH solutions.

2.3. Preparation of Tooth Enamel Specimens. Twenty freshly collected bovine enamel anterior teeth were used to evaluate the acid resistant properties of EB@TiO_2. The collected teeth were subsequently cleaned and disinfected in 10% chloroxylenol solution. Enamel specimens measuring 5 mm × 5 mm × 3 mm was prepared after cutting off the roots using a



(a) (b)

Figure 2: TEM image of (a) EB@TiO_2 composite; (b) particle size distribution.

Table 1: Toothpastes used and their respective pH and buffering characteristics.

Toothpastes	Brand name	Manufacturer	pH in deionized water	Buffering capacity B (mmol 2^{-1} pH^{-1})
Colgate	CALCI™—SEAL protection	Colgate-Palmolive Co	9.61	6.6 ± 02.9
Sensodyne	Rapid relief	GlaxoSmithKline	7.41	2.3 ± 0.02
EB@TiO2	N/A	Researcher	9.31	7.3 ± 0.11

low-speed diamond saw under water cooling conditions. Before subjecting the specimens to acidic condition, the specimens were embedded in a resin (FT 160; AMT composite), and a silicone mold (Blue mold; Agar scientific) was used to make a mounting base. The embedded specimens were randomly assigned into five groups ($n = 4$) and were then exposed to 0.01 mol·L^{-1} HCI (pH = 2) for 30 min as follows: Group 1: unexposed tooth enamel; Group 2: exposed tooth enamel + HCI; Group 3: exposed tooth enamel + HCI + Colgate; Group 4: exposed tooth enamel + HCI + Sensodyne; and Group 5: exposed tooth enamel + HCI + EB@TiO_2.

After 30 min of acid exposure, the specimens were rinsed in deionized water for 30 s and blot-dried.

2.4. Atomic Force Microscopy (AFM) Analysis. AFM (Nanoscope; Bruker) was used to analyze the mean square roughness (R_{rms}) values of the specimens. The instrument was set in contact mode with a scanning size of 10 μm and a scan rate of 2.394 Hz. For each specimen, 5 different measurements of the R_{rms} values were made. The average measured R_{rms} values were then used for the statistical analysis.

2.5. Field-Scanning Electron Microscope Evaluation of the Specimens. The surface of the exposed and unexposed specimens was characterized using scanning electron microscope (FESEM; Carl Zeiss). As a proxy measure, a specimen from each group was analyzed microscopically.

2.6. Raman Spectroscopy Analysis of Specimens. The changes in the mineral content of the specimens were observed using a Raman (Perkin Elmer *precisely* Raman-station 400). The Raman analysis was done on the all the samples with the laser power set at 70 mW, exposure every 10 seconds for 3 seconds at a time. Five different measurements were done for each samples and the average was used for statistical analysis.

2.7. Statistical Analysis. The mean roughness (R_{rms}) value of the specimens was evaluated with 1-way analysis of variance (ANOVA) by means of statistical software (IBM SPSS Statistics v25; IBM Corp.), followed by Bonferonni correction and the significance level was set at $\alpha = 0.05$. For Raman analysis, the base line correction was estimated by the polynomial fitting method, and the peak area and height were determined from the Gaussian plot using Origin Pro software (OriginLab Corporation v 8).

3. Results

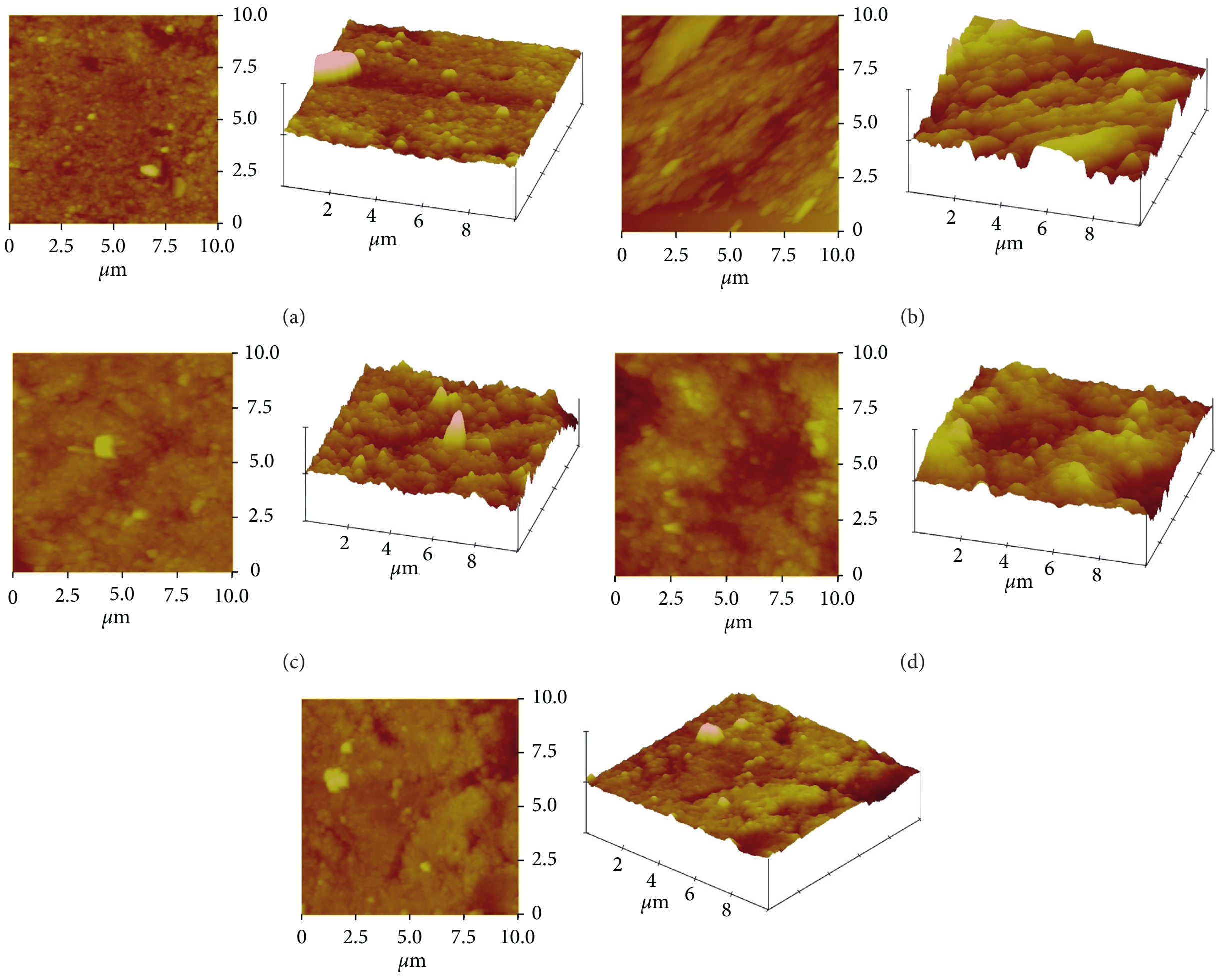
3.1. Atomic Force Microscopy (AFM). The 1-way ANOVA, mean, standard deviation, and standard error results are shown in Table 2. A notable statistical difference was observed in the mean surface roughness (R_{rms}) for the unexposed and exposed specimens ($P < 0.05$).

Comparing the unexposed specimens (group 1) with specimens exposed to HCI alone (group 2), a significant difference were measured ($P < 0.05$). The unexposed specimens had the lowest mean R_{rms} (32.6 ± 16.3 nm) values while specimens exposed to HCI alone had the highest value (101.9 ± 18.0 nm). No significant differences were measured for the unexposed specimens and the specimens exposed in groups 3, 4, and 5, respectively ($P > 0.05$). AFM micrograph shown in Figure 3 further illustrates the surface profile of the specimens. The surface roughness appeared more pronounced for Figures 3(b)–3(d) when compared against Figures 3(a) and 3(e).

Table 2: Mean surface roughness, standard deviation, standard error, and ANOVA.

Groups	N	Mean ± SD	Std. error	95% confidence interval for mean		P	Post hoc bonferroni test
				Lower bound	Upper bound		P
Unexposed tooth	4	32.6 ± 16.3 nm	8.1	6.7077	58.5478		$0.02^{1,2}$
Exposed tooth + HCI	4	101.9 ± 18.0 nm	9.0	73.1958	130.5232		$0.295^{1,5}$
Exposed tooth + HCI + Colgate	4	65.2 ± 29.0 nm	14.5	19.0787	111.4123	0.021	$0.992^{1,3}$
Exposed tooth + HCI + Senosdyne	4	83.1 ± 33.7 nm	16.8	29.4663	136.6767		$0.159^{1,4}$
Exposed tooth + EB@TiO_2	4	57.2 ± 29.6 nm	14.8	10.0747	104.3583		$1.00^{1,5}$

Superscript numbers indicate significant differences between the sample groups (ANOVA, $P < 0.05$).



(a) (b) (c) (d) (e)

Figure 3: AFM profile of (a) unexposed tooth; (b) after exposure to HCI; (c) after exposure to HCI + Colgate toothpaste; (d) after exposure to HCI + Sensodyne; (e) after exposure to HCI + EB@TiO_2.

3.2. Field-Scanning Electron Microscope Observation of Specimens. The FESEM images of the specimens are shown in Figure 4. The images revealed surface differences between the unexposed specimens, exposed tooth alone, and the test groups (EB@TiO_2 and commercial toothpastes). While using the unexposed specimen to benchmark the demineralization of the exposed sample groups, the images in Figures 4(b) and 3(d) visibly showed evidence of the prismatic destruction of the hydroxyapatites which suggest erosion of tooth specimens exposed to acid alone and the Sensodyne toothpaste. In contrast, the tooth specimen exposed to acid in the presence of both EB@ TiO_2 and Colgate showed less evidence of the prismatic destruction of the enamel (Figures 4(c) and 3(e)).

3.3. Raman Spectroscopy. Furthermore, the changes in the position of phosphates (V_3 ($PO_4{}^{3-}$)) peaks for the unexposed and exposed specimens are given in Figure 4. In the unexposed specimens, the peak was only slightly prominent

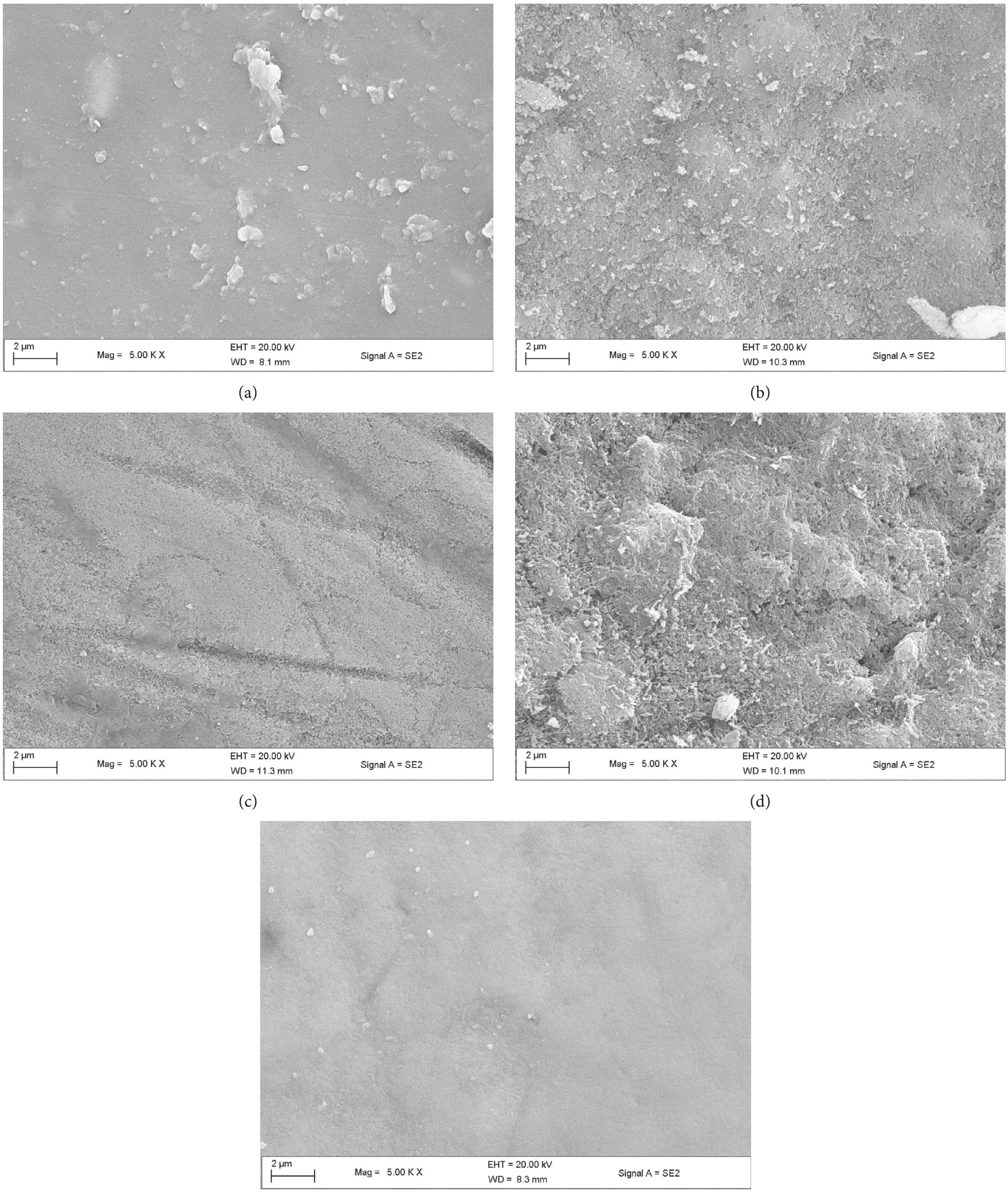


Figure 4: FESEM images of (a) unexposed tooth; (b) after exposure to HCI; (c) after exposure to HCI + Colgate toothpaste; (d) after exposure to HCI + Sensodyne; (e) after exposure to HCI + EB@TiO_2.

(Figure 5(e)). On the other hand, the peaks for the specimen exposed in HCI alone (Figure 5(a)) were distinctly prominent to a considerable height (Table 3). Moreover, only slight changes were noticed in the peaks of the specimens exposed to sample groups 3 (Figure 5(c)), 4 (Figure 5(b)), and 5 (Figure 5(d)), respectively.

4. Discussion

The purpose of this study was to examine the protective effect of a modified eggshell-titanium dioxide composite (EB@TiO_2) in comparison with some commercial toothpastes against erosive acids. In line with suggested

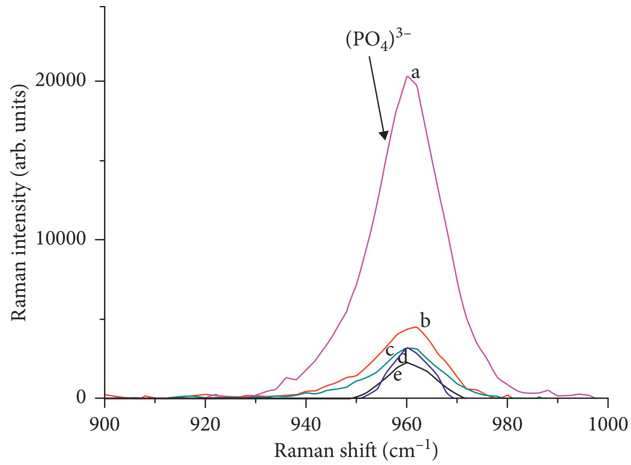


Figure 5: Raman spectrum obtained for tooth samples (a) expose to HCI alone; (b) expose to HCI + Sensodyne; (c) exposed HCI + Colgate toothpaste; (d) expose to HCI + EB@TiO_2; (e) unexposed tooth.

Table 3: Peak analysis as determine by the Gaussian plot.

Sample groups	Peak parameters	
	Height	Area
Unexposed tooth	2454.2	32769.3
Tooth exposed to HCI	18853.3	345765.1
Tooth exposed to HCI + Colgate toothpaste	3956.9	52589.4
Tooth exposed to HCI + Sensodyne	4270.3	73357.7
Tooth exposed to HCI + EB@TiO_2	2965.9	49185.4

techniques in the literature [22–24], AFM was used to characterize and measure the changes in the surface roughness pre- and postexposure to HCI. The average mean square roughness values (R_{rms}) measured were used for statistical analysis. The R_{rms} is the standard deviation height obtained from the AFM images (Figure 3) in areas of $30 \times 30\,\mu m^2$ with a resolution of 256×256 pixels. The R_{rms} data demonstrated that exposure of tooth enamel to HCI significantly affected the surface roughness ($P < 0.05$).

More so, the R_{rms} values measured for the enamel tooth specimens exposed to HCI alone group were significantly higher than the unexposed tooth enamel ($P < 0.05$). This strongly confirmed that the exposure of enamel to acidic substances leads to enamel demineralization. Whilst tooth enamel comprises mostly of calcium (Ca^{2+}), phosphates (${PO_4}^{3+}$), and hydroxide (OH^-), the enamel is constantly in equilibrium with the surrounding saliva and enamel crystals. In support of Shellis et al. [6], the exposure of the tooth enamel to HCI alone may cause enamel to release more ions to the environment to attain a new state of equilibrium. Consequently, and consistent with Lussi and Carvalho [7], the acidic condition exacerbated the process leading to enamel demineralization (Figure 4(b)).

This notwithstanding, the R_{rms} values measured for the enamel tooth specimen exposed to Colgate, Sensodyne, and EB@TiO_2 were not different from the unexposed tooth ($P > 0.05$). In light of these, it can be inferred that the toothpastes (Colgate and Sensodyne) and test group (EB@TiO_2) were protective against enamel demineralization. This supports the notion that the sampled toothpastes are effective and accessible vehicles to improve enamel resistant against erosive oral environment [9]. In comparing the protective effect of the sampled toothpastes samples against EB@TiO_2, the R_{rms} values with the highest mean was for Sensodyne and the lowest for EB@TiO_2 (Table 2). This could have been attributed to the presence of Ca and or titanium ions in the exposed tooth in the presence of EB@TiO_2. More so, and given the differences in the mean R_{rms} values between EB@TiO_2 and Colgate toothpaste, the observed differences appear to reflect the buffering capacities of the 2 groups (Table 1). This strongly supports Caneppele et al. [25] that the buffering capacity of acidic concentration is critical in erosive potential of acidic substance, which may influence the dissolution rate of the enamel. Equally, the differences in the R_{rms} values between EB@TiO_2 and Colgate toothpastes may also be associated with the modification of the calcium carbonate constituents of eggshell with titanium dioxide. Hence, the tested hypothesis is partially accepted as the EB@TiO_2 composite effectively protected the tooth enamel against erosion.

Furthermore, the Raman spectrum of the exposed and unexposed tooth enamel observed at a peak of $960\,cm^{-1}$ could be attributed to the symmetrical stretching of the tetrahedron oxygen atoms that surrounds the phosphorus atoms [26, 27]. This result is consistent with Ionita [28] that Raman spectrum of healthy enamel and demineralized tooth is observed at a well-defined peak at $959\,cm^{-1}$ vibrations. Moreover, the highest-Raman spectrum demineralization intensity observed for the exposed tooth alone in HCI was nearly 5-times greater than the intensity measured for the unexposed tooth (Figure 5). In addition, He et al. [29] and Targino et al. [30] theorize that the mineral intensity measured in Raman could be a factor that determines the rate of demineralization of tooth enamel. Significantly, the Raman intensity measured for EB@TiO_2 was comparable to those measured for Colgate toothpaste; however, it was lower than the sampled Sensodyne toothpaste (Figure 5). This observation is consistent with the results of Joiner et al. [16] which show that the addition of calcium and or calcium containing materials into toothpaste improves its resistant against acidic erosion on tooth enamel.

In light of the above findings, the researchers plan to further examine the remineralization potential of EB@TiO_2, particularly in the repair of damaged tooth (enamel and dentine). These studies would help establish the suitability of EB@TiO_2 in the maintenance of oral health particularly as a desensitising toothpaste.

5. Conclusion

In conclusion, this study has demonstrated that toothpastes provide protection to the tooth enamel against erosive substance. Notably, the study has shown that EB@TiO_2 offer better protective covering to the enamel against acid attack.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

No conflicts of interest declared.

Acknowledgments

The authors acknowledge the financial support from the National Research Foundation of South Africa (no. 104824).

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Paper V

Onwubu, S.C., Mdluli, P.S. and Singh, S. 2019. The remineralization potential of a modified eggshell-titanium composite-SEM study. *European Journal of Dentistry.* DOI: 10.4103/ejd.ejd_462_18.

The remineralization potential of a modified eggshell–titanium composite-scanning electron microscope study

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ABSTRACT

Context: Dentin hypersensitivity (DH) is a public health concern and challenge to oral health-care providers. For instance, DH presents a great diffi culty for oral health-care provider to diagnose and effectively manage which could compromise the quality of life of dental patients. **Aims:** This paper reports on the remineralization potential of a modifi ed eggshell–titanium dioxide (EB-TiO_2) composite in the management of DH. **Subjects and Methods:** The prepared composite was further characterized using different techniques such as Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscope (TEM), and fi eld scanning electron microscope (FSEM) to establish the modifi cation. Forty freshly extracted bovine anterior teeth were used to evaluate the dentin tubule remineralization potential of EB-TiO_2. Each tooth was sectioned mesiodistally to obtain dentin blocks. The dentin blocks were subsequently agitated in 4% wt citric acid for 2 min to simulate sensitive tooth. Each dentin blocks was randomly assigned to five groups, namely Group 1: untreated, Group 2: EB alone, Group 3: EB-TiO_2 treated, Group 4: Colgate Sensitive treated, and Group 5: Sensodyne (n = 8) which were then subjected to remineralization protocol. **Results:** Both the XRD and FTIR images confi rm the surface medication of EB-TiO_2. The TEM revealed a nonhomogeneous structure with an average particle size of 65 nm. FSEM further was used to observe the remineralization capabilities of the samples. The FSEM image of the dentin specimens treated with EB-TiO_2 shows complete remineralization of the dentin tubules which remain intact postacidic exposure. **Conclusions:** This study confi rmed that EB-TiO_2 composite effectively remineralizes dentin tubules. More so, the composite could be a cheaper and more effi cient therapy material in the management of DH.

Ke ywords: Dentin hypersensitivity, eggshell powder, remineralization, titanium dioxide

INTRODUCTION

Dentin hypersensitivity (DH) is a relatively common complaint affecting >43% of adult's population worldwide.[1] According to the Canadian advisory board on DH,[2] DH is characterized by distinctive short, sharp pain arising from exposed dentinal

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How to cite this article: Onwubu SC, Mdluli PS, Singh S, Bharuth V. The remineralization potential of a modified eggshell–titanium composite-scanning electron microscope study. Eur J Dent 2019;13:XX-XX.

DOI: 10.4103/ejd.ejd_462_18

tubules, particularly in response to external stimuli that are typically thermal, evaporative, tactile, electrical, osmotic, or chemical changes which cannot be ascribed to any other form of dental defects or pathology. Concerning to oral care providers, DH negatively affects the quality of life for dental patients, as they become noncompliant with specific at home-care recommendations such as toothbrushing, thereby contributing to other oral care diseases.[3]

Of interest, to effectively control and manage DH, several theories have been proposed in the last decades to explain the mechanism of DH. Currently, the hydrodynamic theory proposed by Brannstrom is widely regarded as the accepted theory.[4] Hence, approaches to control the hydrodynamic mechanism have resulted in the development of two class of products, namely (1) occluding products or agents that reduce fluid flow within the dentin tubules and (2) agents or products that interfere with the transmission of nerve impulses.[3,5] As the first-line therapy in the treatment of DH, Yang *et al*.[6] advised using an occluding agent to physically block exposed dentin tubules.

Furthermore, occluding agents such as potassium oxalate,[7] sodium fluoride,[8] strontium salt,[1] amorphous calcium phosphate containing casein phosphopeptide,[9] and calcium glycerophosphate[10] have been widely utilized in desensitizing paste for their dentin tubule occluding capabilities. However, the tubules occluded by some of these materials are reported to be superficial with limited infiltration depth.[11] Consequently, patients experience relapse of pain as the occluded tubules are readily re-exposed in an acidic condition.[12] Bearing these in mind, the development of a desirable biomaterial for DH becomes highly critical not only to efficiently occlude the exposed dentin tubules but also remain effective in an acidic environment.

Significantly, eggshells (EBs) have been investigated in recent years for their remineralization capabilities.[13] For instance, Haghgoo *et al*.[14] reported that EB has a rich bioavailable calcium content which could favor the remineralizing of carious lesions. Cutler[15] suggested that nano-sized titanium dioxide (TiO_2) and dental abrasive agents can be used together in occluding open dentinal tubules to effectively reduce DH. Another study[16] demonstrated that nano-sized TiO_2 can improve the acid-resistant properties of calcium carbonates. Given the desirable properties of TiO_2 and the remineralization potentials of EB, a new EB-TiO_2 composite, will be highly important for treating DH. Therefore, however, there is limited evidence in the use of EB-TiO_2 for occluding dentin tubules. The present study aimed to evaluate the remineralization characteristics of EB-TiO_2.

SUBJECTS AND METHODS

Preparation of eggshell–titanium dioxide composite

Modifi cation of EB with TiO_2 was achieved in two steps. In the fi rst step, EBs were ball-milled by placing 30 g of the EB in a 500-mL stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10-mm diameter and dry-milled in a planetary ball mill (Retsch® PM 100) at 400 rpm for 20 min. The collected powder was sieved to a particle size of ≤25 μm using a mechanical sieving shaker (Retsch AS 200, Germany). The EB powder and TiO_2 mixing ratio were optimized following the procedure reported by Ling et al.;[17] 20 g of the fi ne EB powder obtained in Step 1 was modifi ed by adding 5 g of anatase TiO_2 (≤15 μm). The mixture was subsequently ball-milled for 100 min to obtain EB-TiO_2 composite.

Characterization of eggshell–titanium dioxide

Fourier-transform infrared spectroscopy analysis

The infrared spectra were measured using a Perkin-Elmer Universal Attenuated Total Reflectance spectrometer to identify the functional group constituents of EB-TiO_2.

X-ray diffraction analysis

The X-ray diffraction (XRD) analysis was performed to observe the possible changes in crystallinity between the EB powder, TiO_2, and EB-TiO_2. The XRD patterns were recorded using a diffractometer (PANalytical-Empyrean instrument; Co radiation: 1.54056 A°) and analyzed between 0° and 90° (2 theta). The voltage, current, and pass time used were 40 Kv, 40 mA, and 1 s, respectively. Any reference to this?

Energy-dispersive X-ray spectroscopy and scanning electron microscope analysis

Energy-dispersive X-ray (EDX) spectroscopy was used in conjunction with a scanning electron microscope (SEM) (Field Emission, Carl Zeiss, Germany) operating at controlled atmospheric conditions at 20 kV to examine the surface morphology and the elemental composition of the EB-TiO_2. Before SEM observation, the surface was coated with a thin, electric conductive gold film to prevent the buildup of electrostatic charge.

Transmission electron microscopic analysis

A transmission electron microscope (TEM) was used to observe the particle size, shape, and distribution of TiO_2-EB. Very small quantities of EB-TiO_2 were dispersed in 10-ml ethanol and sonicated at 10kv for 10 min. Subsequently, thin cross sections of cryomicrotomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids. The analysis was conducted using a (TEM, Philips CM 120 model) at 120 kV.

Preparation of dentin tooth specimens

Forty freshly extracted bovine enamel anterior teeth were obtained from a slaughterhouse, South Africa. The collected teeth were subsequently cleaned and disinfected in 10% chloroxylenol solution. Dentin specimens measuring 5 mm × 5 mm × 1 mm were prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water-cooling conditions. Thereafter, the prepared dentin specimens were wetly grounded with silicon carbide polishing papers (600–1000 grits) for 60 s. Before simulating the sensitive model, the specimens were mounted in a resin (Advanced Materials Technology Composite, South Africa). The dentin tubules were opened by soaking the specimens in 4% wt citric acid solution for 2 min to simulate sensitive tooth. Each dentin block was randomly assigned to five groups, namely Group 1: untreated, Group 2: EB alone, Group 3: EB-TiO_2 treated, Group 4: Colgate Sensitive treated, and Sensodyne (n = 8) which were then subjected to remineralization protocol.

Evaluating in situ the remineralization and acid-resistant characteristics of eggshell–titanium dioxide

In evaluating the remineralization potential of EB-TiO_2, the prepared dentin tooth specimens were agitated in a beaker containing 1 g of the synthesized EB-TiO_2 and 40-mL deionized water for 3 hours . The specimens were subsequently rinsed and blot-dried. The treated specimens were further exposed to 4% wt citric acid solution for 2 min. After exposure, the specimens were rinsed with deionized water and blot-dried.

Energy-dispersive X-ray spectroscopy and scanning electron microscope analysis

EDX spectroscopy was used in conjunction with SEM (Field Emission, Carl Zeiss, Germany) operating at controlled atmospheric conditions at 20 kV to examine the surface morphology and the elemental composition of the occluded dentin pre- and posttreatment and postacidic exposure. Before SEM observation, the surface was coated with a thin, electric conductive gold fi lm to prevent the buildup of electrostatic charge.

RESULTS

Characterization

The Fourier-transform infrared (FTIR) spectra of EB powder, TiO_2, and EB-TiO_2 is presented in Figure 1. The band aspect of the FTIR spectra shows the difference between the EB powder [Figure 1a], TiO_2 [Figure 2b], and EB-TiO_2 [Figure 2c]. Although both EB powder [Figure 1a] and EB-TiO_2 [Figure 1c] exhibit absorption peaks of carbonates at 1411/cm, the broad stretching displayed for EB-TiO_2 below 1000/cm suggest the surface medication of calcium carbonate structure in EB powder with TiO_2.[18]

The XRD pattern of EB powder, TiO_2, and EB-TiO_2 is presented in Figure 2. For Figure 3a, the characteristic peak marked around 34.5º (2 θ) indicates the presence of thermodynamically stable calcite crystalline of the carbonate constituent in EB powder.[18] In Figure 3b, the XRD pattern of pure TiO_2 shows a characteristic diffraction peak with values lying at 2 θ =29.5° that corresponds to anatase phase and is confirmed with the International Centre for Diffraction Data (ICDD Ref: 98-009-6946). The EB-TiO_2 diffraction peak shown in Figure 3c revealed the presence of calcite and anatase crystalline structure. More importantly, and supporting the FTIR spectroscopy results, the XRD pattern of EB-TiO_2 exhibits a single-phase formation of the carbonate structure. This may likely be due to the balling time used. It is reported in the literature that

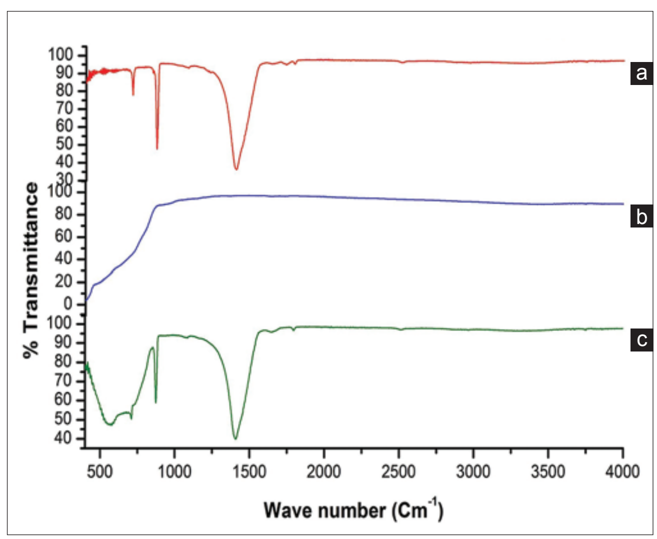

Figure 1: Fourier-transform infrared spectra are showing (a) eggshell powder. (b) Titanium dioxide. (c) Eggshell–titanium dioxide

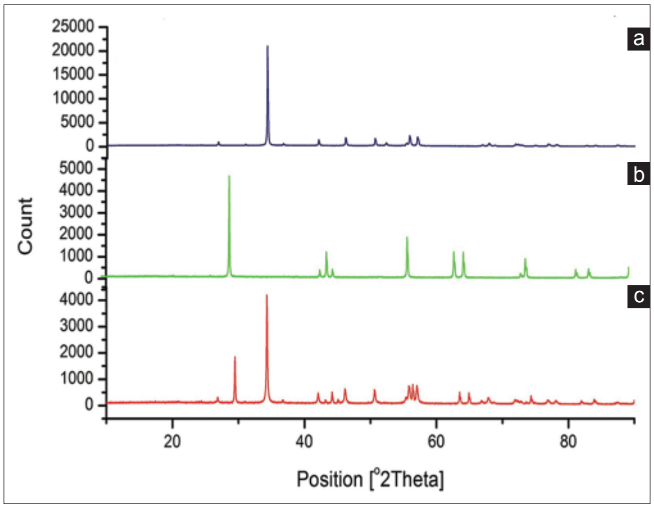

Figure 2: X-ray diffraction pattern of (a) eggshell powder. (b) Titanium dioxide. (c) Eggshell–titanium dioxide

calcium carbonate undergoes phase transformation on milling above 250 min.[19]

The SEM images of the modified EB-TiO_2 and the elemental mapping are illustrated in Figure 3. As presented in Figure 3a, the SEM images show that approximately irregular and spherical particles coexisted. The irregular-shaped particles are indicative of EB powder, whereas the spherical particles typified the TiO_2 particles. It can be observed that the pure TiO_2 particles were scattered on the surface of the composite [Figure 3a]. More so, it can be seen that TiO_2 sufficiently coated the EB powder particles and formed a compact layer of TiO_2 firm. The said surface coating of TiO_2 on the EB powder is further confirmed by the EDX mapping [Figure 3b].

The EDX spectrum for the EB-TiO_2 is shown in Figure 4. The high level of calcium, oxygen, and carbon could be attributed to the calcium carbonate constituent of EB powder. In addition, titanium is evidently present which confirm the surface modification of EB powder. This result correlates with the FTIR analysis in Figure 1.

The TEM image of EB-TiO_2 is shown in Figure 5. Evidently, the TEM image revealed a nonhomogeneous structure of spherical-shaped particles and irregular particles with different sizes of distribution. Further to this, nanoparticles and some submicron particles were observed in the TEM images. The presence of submicron particles may be attributed to the commutation time used in achieving the modification of the composite.

Remineralization

The SEM micrograph of dentin tooth after abrading with silicon carbide for 60 s and subsequently exposed to 2%

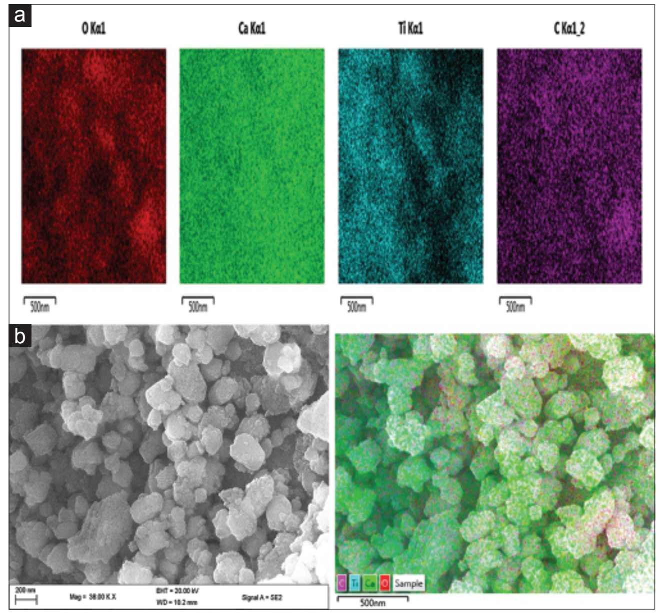

Figure 3: Scanning electron microscope micrograph for (a) Eggshell–titanium dioxide. (b) Energy-dispersive X-ray mapping

wt citric acid for 2 min is given in Figure 6. The dentin tubules were hardly exposed after abrasion with silicon carbide for 60 s [Figure 6a]. In contrast, after exposure to 2% wt citric acid for 2 min, the dentin tubules were evident with increased diameter [Figure 6b].

The SEM images of the dentin specimens treated with EB powder, EB-TiO_2, Colgate, and Sensodyne are seen in Figure 7. The images reveal occlusion differences between the EB-TiO_2-treated specimens with the other test groups (EB powder, Colgate, and Sensodyne). The dentin specimen treated with EB-TiO_2 showed that nearly all the tubules are closed or remineralized with a compact surface [Figure 7b]. In contrast, and as shown in Figure 7a, c, and d, open tubules were visible on the dentin specimen treated with EB powder, Colgate, and Sensodyne, respectively.

Added to the above, the SEM micrograph of dentin specimen's treatment EB-TiO_2 and postexposure to citric acid is given in Figure 8. Figure 8a visibly confi rmed the remineralization of the dentinal tubules. Equally important, particles of EB-TiO_2 deposits could be observed in the image at higher magnification, which suggests that the occluding or sealing of the tubules had depth and penetration. The EDX further confi rmed Ti peaks for TiO_2 [Figure 8a1]. The SEM image in Figure 8b revealed that the occluding bond remains intact after exposure to acid showing strong resistant to demineralization. This is also confirmed by the EDX spectrum [Figure 8b1].

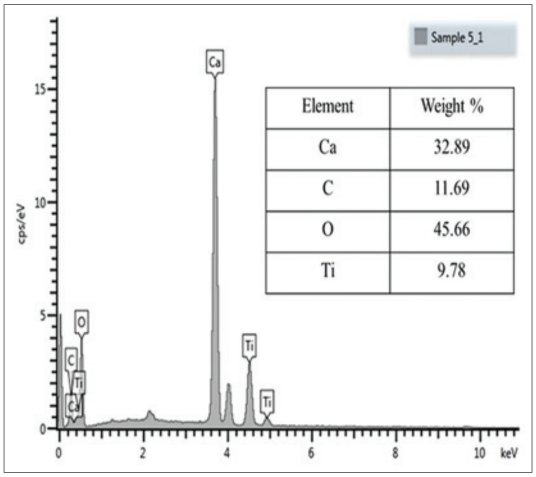

Figure 4: Energy-dispersive X-ray spectrum of eggshell–titanium dioxide

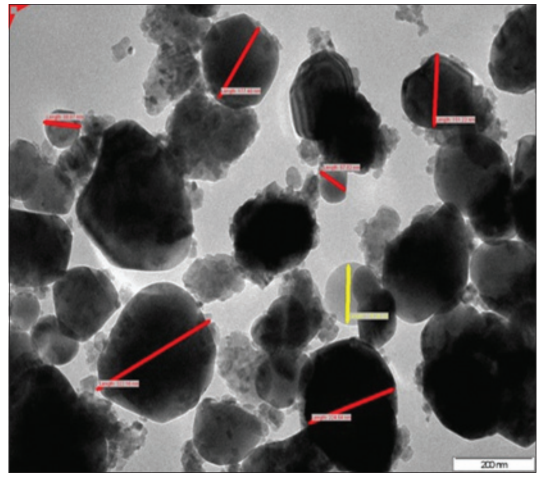

Figure 5: Transmission electron microscope images eggshell–titanium dioxide

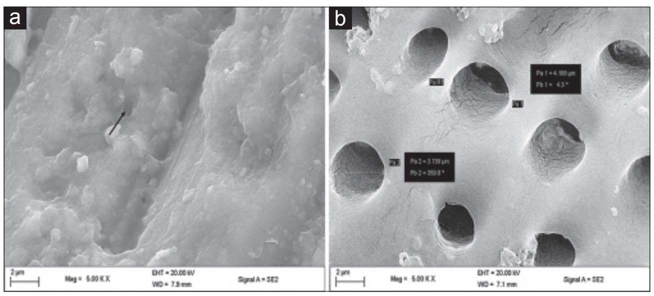

Figure 6: Representative scanning electron microscope micrograph for (a) dentin surface wet ground with silicon carbide paper for 60 s. (b) Pretreatment of dentin surface exposed 4% wt citric acid solution for 2 min (×5000). Arrows showing narrow dentin tubules

DISCUSSION

The purpose of this study was to evaluate *in situ* the remineralization characteristics of a modified EB powder and TiO_2 composite (EB-TiO_2). The modification of EB-TiO_2 was achieved using a planetary ball-milling procedure. This technique is common mechanochemistry principle of producing fine powder in many different fields.[20] The characterization results confirm that EB-TiO_2 was successfully modified and the particle sizes obtained were in nano dimension [Figure 5]. This finding is consistent with Tsai *et al*.[20] that planetary ball milling reduces particles to fine powder through the imposition of impact and frictional forces. More importantly, the presence of both carbonate structure and Ti-O-Ti stretching in the EB-TiO_2 composite confirmed that the ball milling did not negatively impact on the carbonate composition of the EB powder [Figure 1].

Although EB powder is predicted to be the future of tooth remineralization,[13] the SEM images of the treated

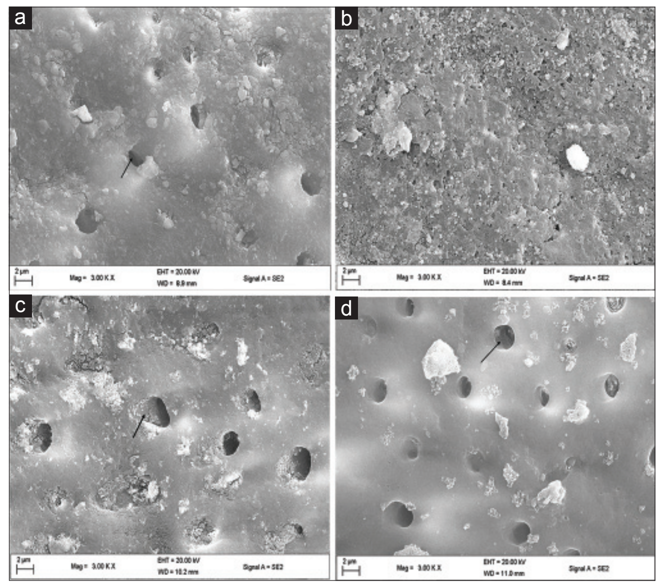

Figure 7: Representative scanning electron microscope micrograph for dentin surface treated with (a) eggshell powder. (b) Eggshell–titanium dioxide. (c) Colgate. (d) Sensodyne (agitated for 3 h, ×3000). Arrows showing exposed tubules

specimens suggest that modification of EB powder and TiO_2 would be more effective in occluding dentin tubules [Figure 7]. Significantly, the dentin specimens treated with EB-TiO_2 demonstrate outstanding seal as well as acid-resistant characteristics [Figure 8]. This is in agreement with Cutler[15] suggestion that nano-sized TiO_2 and dental abrasive agents can be used together in occluding open dentinal tubules to effectively reduce DH. Moreover, the presence of TiO_2 coating on the surface of EB-TiO_2 may have contributed to the occlusion remaining effective in an acidic environment [Figure 8b]. It can, therefore, be suggested that EB powder modified with TiO_2 could potentially block open dentinal tubules while remaining intact in an acidic condition. This is in agreement with the report of Tao *et al*.[16] that TiO_2

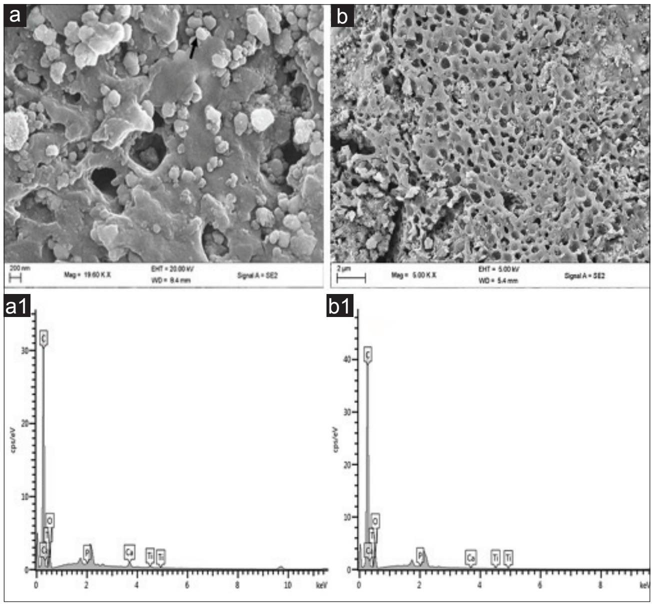

Figure 8: Representative scanning electron microscope micrograph for (a) dentin surface after treatment with eggshell–titanium dioxide for 3 h.
(b) Dentin surface postexposure to 4 wt% citric acid solution for 2 min (a, ×19000; b × 5000). (a1) Energy-dispersive X-ray spectrum of the dentin surface treated with eggshell–titanium dioxide; energy-dispersive X-ray. (b1) spectrum of the dentin surface postexposure to 4 wt% citric acid solution for 2 min. Arrows pointing to eggshell–titanium dioxide particles

can improve the acid-resistant properties of calcium carbonates.

While several previous studies[3,11,21] have reported on the remineralization and occluding potentials of Colgate and Sensodyne toothpastes, the findings from this study reveal that the occluding abilities of the tested toothpaste were inferior when compared to that observed for EB-TiO$_2$ [Figure 7]. This could, however, be attributed to the brand of toothpaste tested. According to Schiff *et al*.,[22] desensitizing toothpastes such as Sensodyne and Colgate toothpastes are originally designed to deliver potassium ions that act as nerve-depolarizing agents in the treatment of DH. However, a new brand of Colgate, particularly calcium carbonate–arginine combination, has shown promising results in occluding dentin tubules.[21] In light of the above findings, further studies are planned to examine clinically, the effectiveness of EB-TiO2 in comparison with other brands of desensitizing toothpaste. These studies should help validate the suitability of EB-TiO2 as a composite for the management of DH.. These studies should help validate the suitability of EB-TIO$_2$ as a composite for the management of DH.

CONCLUSIONS

In summary, both the XRD and FTIR results confirmed the successful modifi cation of EB-TiO$_2$. The high-resolution TEM revealed a nonhomogeneous powder with an average particle size distribution of 65 nm. Furthermore, the FESM images suggest the EB-TiO$_2$ composite could effectively remineralize and occlude dentinal tubules. It was also demonstrated that the remineralization capabilities of the composite were intact postacidic exposure. Importantly, EB-TiO$_2$ could provide a cheaper and efficient therapy material in the management of DH management. This could go a long way in reducing the global burden of waste in the environment.

Mechanism explain what happened nerve depolarization or hydrodynamic theory.

Also what about seal and protect and other agents available in the market?

What is there role and constituents?

Financial support and sponsorship
The authors acknowledged the financial support from the National Research Foundation of South Africa (No. 104824).

Conflicts of interest
There are no conflicts of interest.

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Paper VI

Onwubu, S.C., Mdluli, P.S., Singh, S. and Tlapana, T. 2019. A novel application of nano eggshell/titanium dioxide composite on occluding dentine tubules: An in vitro study. *Brazilian Oral research*, 30(16): 1-10. http://dx.doi.org/10.1590/1807-3107bor-2019.vol33.0016

Original Research
Dental Materials

A novel application of nano eggshell/titanium dioxide composite on occluding dentine tubules: an *in vitro* study

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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https://doi.org/10.1590/1807-3107bor-2019.vol33.0016

Submitted: September 4, 2018
Accepted for publication: January 10, 2019
Last revision: January 17, 2019

Abstract: To synthesize Nano eggshell-titanium-dioxide (EB@TiO2) biocomposite and to evaluate its effectiveness in occluding opened dentine tubules. EB@TiO2 was synthesized and characterized using X-ray diffraction (XRD), and Transmission Electron Microscope (TEM). Sixteen simulated bovine dentine discs were prepared and randomly assigned into four groups according to the following treatment (n = 4): Group 1: No treatment; Group 2: eggshell powder; Group 3: EB@TiO2; Group 4: Sensodyne. These were then agitated in a solution of 1g powder and 40mL water for 3hours. Thereafter, each dentine discs from the respective groups were post-treated for 5 min with 2wt% citric acid to test their acid resistant characteristics. Scanning Electron Microscope (SEM) was used to observe the effectiveness of occluded dentine pre- and post-treatment. The cytotoxicity of the synthesized EB@TiO2 was tested using NIH 3T3 assay. ANOVA was used to evaluate the mean values of the occluded area ratio and the data of MTS assay. This was followed by a multi-comparison test with Bonferroni correction ($\alpha = .05$). The XRD confirmed that EB@TiO2 was successfully modified through ball-milling. The TEM revealed the presence of both spherical and irregular particle shape powders. The SEM result showed that EB@TiO2 could effectively occlude open dentine tubules. Equally, the result demonstrated that EB@TiO2 exhibited the highest acid resistant stability post-treatment. NIH 3T3 assay identified that EB@TiO2 had little effect on the NIH 3T3 cell line even at the highest concentration of 100µg/ml. This study suggests that the application of EB@TiO2 effectively occluded dentine tubules and the occlusion showed a high acid resistant stability.

Keywords: Dentin; Dentin Sensitivity; Dentin Desensitizing Agents; Tooth Remineralization.

Introduction

Dentine Hypersensitivity [DH] is a common occurrence and under extreme conditions when dentine tubules is exposed to the oral cavity patients will experience a short, sharp pain.[1] From a public health perspective, DH is a significant oral health concern affecting more than 43% of adult's population worldwide. [2] As a consequence, DH if left untreated will negatively affect the quality of life for dental patients'. In particular, and as Schiff et al.[3] points out, DH sufferers tend to modify their habits by eliminating certain foods and drinks from their regular diets and they become non-complaint with specific at-home care recommendations such as tooth brushing. Consequently, caries formation, gingival inflammation and periodontal problems are likely to increase. Yang et al.[4] have therefore advised using an occlusion agents to physically block exposed dentine tubules.

Over the last decades, the oral care industry have witnessed proliferation of different occlusion materials for treating DH. Among these materials, the use of potassium oxalates,[5] sodium fluoride,[6] strontium salt,[2] amorphous calcium phosphate containing casein phosphopeptide;[7] calcium glycerophosphate,[8] and calcium carbonates (mainly as abrasive agents)[9] have gain significant interest as an occlusion materials. Although the aforementioned occlusion materials have been reported to provide some relief to patients, the dentine tubules occluded by some of these materials are reported to be superficial with limited infiltration depth-which could be readily re-exposed in an acidic environment.[10] Thus, short-living the treatment effects and subsequently resulting to DH relapse.[11] Given the limited drawbacks, developing a desirable biomaterials for DH becomes highly critical to not only efficiently occlude the exposed dentine tubules, but also remain effective in acidic environment.

Of interest, eggshells are currently being investigated for their remineralization capabilities.[12] More recently, Haghgoo et al.[13] reported that eggshell has a rich bioavailable calcium content, which favours the remineralizing of caries lesions. Cutler[14] suggested that nanosized titanium dioxide and dental abrasive agents can be used together in occluding open dentine tubules. Other scholar have indicated that nanosized titanium dioxide can improve the acid resistant properties of calcium carbonates.[15] Given the desirable properties of titanium dioxide (TiO_2) and the remineralization potentials of eggshells (EB), a new EB@TiO_2 bio composite will be highly important for treating DH. Despite the enormous potential of eggshell-titanium dioxide bio composite, there is limited evidence in its use for occluding dentine tubules. The study aimed to synthesize Nano eggshell-titanium-dioxide (EB@TiO_2) bio composite and evaluate its effectiveness in occluding opened dentine tubules. The hypothesis tested was that EB@TiO_2 will effectively occlude open dentine tubules and the occlusion show more acidic resistant.

Methodology

Preparation of Eggshell-Titanium dioxide composite (EB@TiO_2)

Modification of eggshell with titanium dioxide was achieved in two steps. In the first step, eggshells were ball-milled by placing 30g of the eggshell in a 500ml stainless jar (inner diameter of 100 mm), together with 10 stainless steel balls of 10mm diameter and dry-milled in a planetary ball mill (Retsch ® PM 100) at 400 rpm for 20 min. The collected powder was sieved to a particle size of ≤ 25µm using a mechanical sieving shaker (Retsch AS 200, Germany). The eggshell powder and titanium dioxide mixing ratio was optimized following the procedure reported by Lin et al.[16] 20g of the fine eggshell powder obtained in step 1 were modified by adding 5g of anatase titanium dioxide (≤ 15µm). The mixture was subsequently ball-milled for 200 min to obtain eggshell-titanium dioxide bio composite.

Characterization of *EB@TiO_2*

X-Ray Diffraction Analysis

The X-ray diffraction (XRD) analysis was performed to observe the possible changes in crystallinity between the eggshell powder, titanium dioxide, and EB@TiO_2. The XRD patterns were recorded using a diffractometer (PANalytical-Empyrean instrument; Co radiation 1.54056 A^o) and analysed between 0-90^o (2 theta). The voltage, current and pass time used were 40 Kv, 40mA and 1s, respectively.

Microscopic analysis

A Transmission Electron Microscope (TEM) was used to observe the particle size, shape and distribution of EB@TiO_2. Very small quantities of EB@TiO_2 were dispersed in 10ml ethanol and sonicated at 10kv for 10 min. Subsequently, thin cross-sections of cryo-microtomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids. Analysis was conducted using a transmission electron microscope (TEM-Philips CM 120 model) at 120 kV.

Specimen preparation and acidic challenge

Sixteen freshly extracted bovine-enamel anterior teeth were obtained from a slaughter house, South Africa. The collected teeth were subsequently cleaned and disinfected in 10% chloroxylenol solution. Dentin discs measuring 5mm x 5mm x 1mm was prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water cooling conditions. Subsequently, the prepared dentin disc was wet grounded with silicon carbide polishing papers (600–1,000 grits) for 60 seconds. Before simulating the sensitive tooth model, the discs were mounted in a resin (AMT composite, South Africa). Silicone mold (Silicone rubber mold; Agar scientific) was used to make a mounting base. A fast setting resin (F160: AMT composite) was mixed in a disposable plastic cup in a 1:1 ratio and poured into the mold. After approximately 2 minutes. The embedded resin was removed from the silicone mold. Thereafter, dentine tubules was opened by soaking the specimens in 1 wt. % citric acid solution for 30 min. The specimens were randomly assigned into four groups (n =4), namely:

a. Group 1: No treatment group.
b. Group 2: Eggshell powder treated group.
c. Group 3: EB@TiO_2 treated group, and
d. Group 4: Sensodyne treated group.

The specimens were agitated in a beaker containing 1g of the synthesized EB@TiO_2 and 40mL deionized water for 3 hours. Similar procedure were followed for eggshell powder and Sensodyne paste. The specimens were subsequently rinse and blot tried. More so, and as a proxy measure, a representative of each sample group were selected to determine the acid resistant characteristics of the treated specimen. This were subsequently exposed to 2wt. % citric acid solution (pH 2) for 5min. After exposure, the specimens were rinsed with deionized water and blot tried.

Scanning electron microscope evaluation of the occluded specimen

Scanning electron microscope (Field Emission-Carl Zeiss) operating at controlled atmospheric conditions at 20 kV was used to evaluate the occluded dentine pre- and post-acidic exposure. Prior to SEM observation, the surface was coated with a thin, electric conductive gold film to prevent build-up of electrostatic charge. In addition, the ratios of occluded tubules was further computed using ImageJ software (National Institute of Health USA, http://imagej.nih.gov./ij). This was calculated by dividing the area of occluded tubules by the total tubules area using x 3000 magnification images (n = 4).

Cytotoxicity assay

Eggshell powder and EB@TiO_2 samples were dispersed in Dimethyl Sulfoxide (DMSO), before the administration to the cell line. An MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used to evaluate change in cell viability. The samples were tested across 2 plates in duplicate (n=6) and the average value reported. NIH 3T3 cells were grown using normal tissue culture techniques. The cells (1 x 105 cells/ml) were incubated in 96 well plates at 37°C overnight, with the subsequent addition of the supplied compounds, in concentrations of (100 µg/ml, 50.0 µg/ml, 25.0 µg/ml, 12.5 µg/ml, 6.25 µg/ml, and 3.13 µg/ml). The cells were left to incubate for 4 days. Thereafter, MTS (5 µl) was added to the cells. The absorbance values were measured at 490 nm after 1h, 2h and 4 hour incubation periods, averaged and the viability curves drawn up.

Statistical analysis

The mean values of the occluded area ratio in SEM study and the % viability data of MTS assay were evaluated with 1-way analysis of variance (ANOVA) using statistical software (IBM SPSS Statistics v24;

IBM Corp). This was followed by a multi-comparison test with Bonferroni correction ($\alpha = .05$).

Results

Characterization

The XRD analysis of eggshell powder titanium dioxide, and the synthesized EB@TiO_2 are presented in Figure 1. For Figure 1A, the characteristic peak marked around 34.5° (2θ) indicates the presence of thermodynamically stable calcite crystalline structure, which is similar to calcium carbonate.[17] The EB@TiO_2 diffraction peak with values lying at $2\theta = 29.5°$ corresponds to anatase phase and is confirmed with International Centre for Diffraction Data (ICDD Ref: 98-009-6946). Consistent with Tao *et al.* [15], the shape, intensity, and location of the EB@TiO_2 peaks corresponding to the anatase

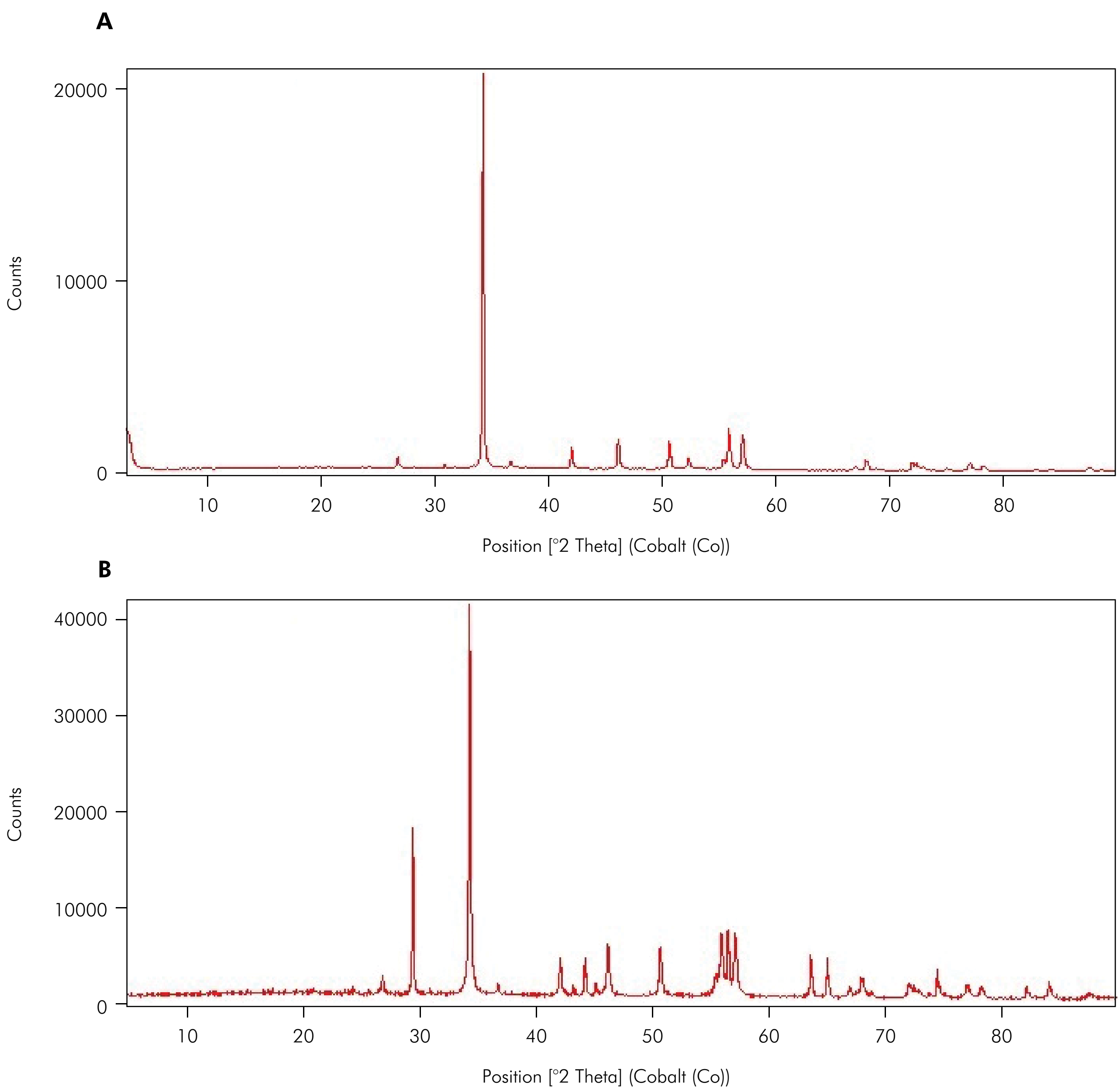

Figure 1. XRD pattern of (A) eggshell powder; (B) EB@TiO_2.

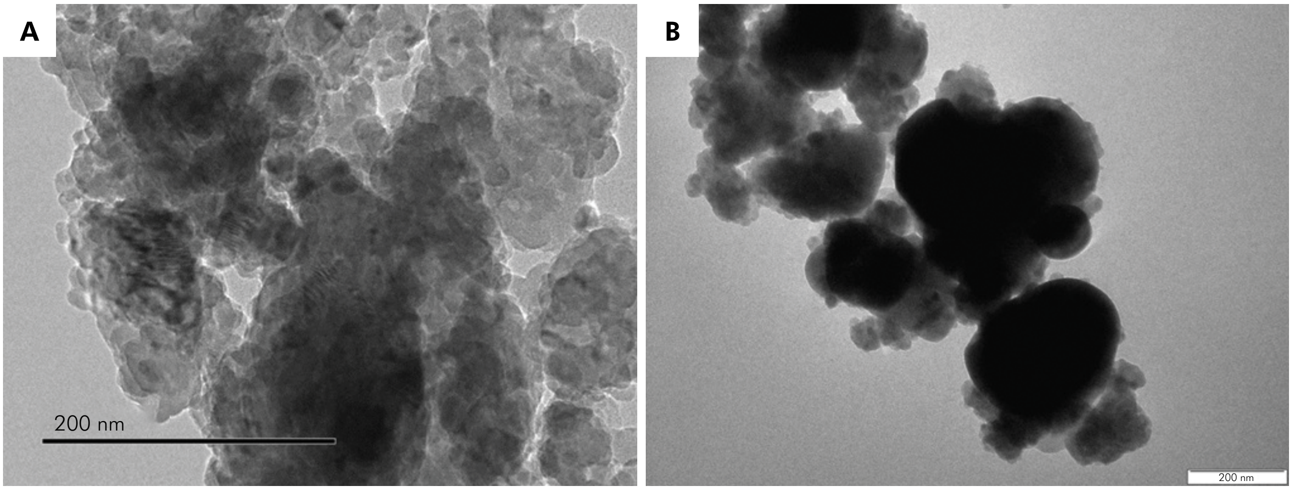

Figure 2. TEM images: (A) eggshell powder; (B) EB@TiO_2.

suggests the deposition of TiO_2 on the surface of the $CaCO_3$.

The morphology, shape and particle size of eggshell powder and EB@TiO_2 were studied using TEM. According to the TEM image in Figure 2 A, an irregular particle shape were notice in the eggshell. In contrast, and as seen in Figure 2 B, the presence and irregular particle shapes coexisted with a spherical shape particles. The presence of the irregular shaped particles is attributed to the calcite shape of the eggshell powder whilst the spherical shaped particles typified the presence of TiO_2.

Cytotoxicity testing

The viability of NIH 3T3 cell lines exposed to different concentrations of eggshell powder and EB@TiO_2 are shown in Figure 3. The samples were tested against NIH 3T3 mouse fibroblasts cell lines. No significant difference was determined among the various groups ($p > 0.05$). Equally, there were no significant differences in all pairwise comparisons amongst the different concentrations ($p > 0.05$).

Observation of the occluded dentine tubules

The 1-way ANOVA, mean, standard deviation, and standard error results are given in Table 1. Notably, the mean ratio of occluded tubules values for the specimens treated with eggshell powder EB@TiO_2, and Sensodyne were statistically different ($p < .001$).

The specimens treated with EB@TiO2 showed highest mean occluded areas (83.25 ± 12.47 μm^2), while the specimen group treated with Sensodyne covered the lowest open tubules (25.00 ± 8.04 μm^2). The results of the post hoc comparison test (Table 2) suggests that area of tubules occluded by EB@ TiO_2 group were significantly higher than those for the eggshell powder group ($p < .001$) and the Sensodyne group ($p < .05$). Equally, statistical differences were found in the occluded tubules for the groups treated with eggshell powder and Sensodyne ($p < .05$).

The SEM micrograph of the occluded dentine tubules pre-and post-treatment in different sample groups are displayed in Figure 4. In group 1 (A1–B1), it was observed that the dentine surfaces were free of smear layer and all the dentine tubules were open after agitation in 1 wt. % citric acid for 30 min. In group 2 (A2–B2), the dentine tubules were partially blocked with some visibly open tubules. In group 3 (A3–B3), the dentine tubules were completely blocked by the particles of EB@TiO_2. In group 4 (A4–B4), most of the dentine tubules still remain open after treatment with Sensodyne. In addition, the SEM image (C2–C4) revealed differences in the treated dentine specimen post-treatment in 2wt % citric acid. For example, and in contrast to the images

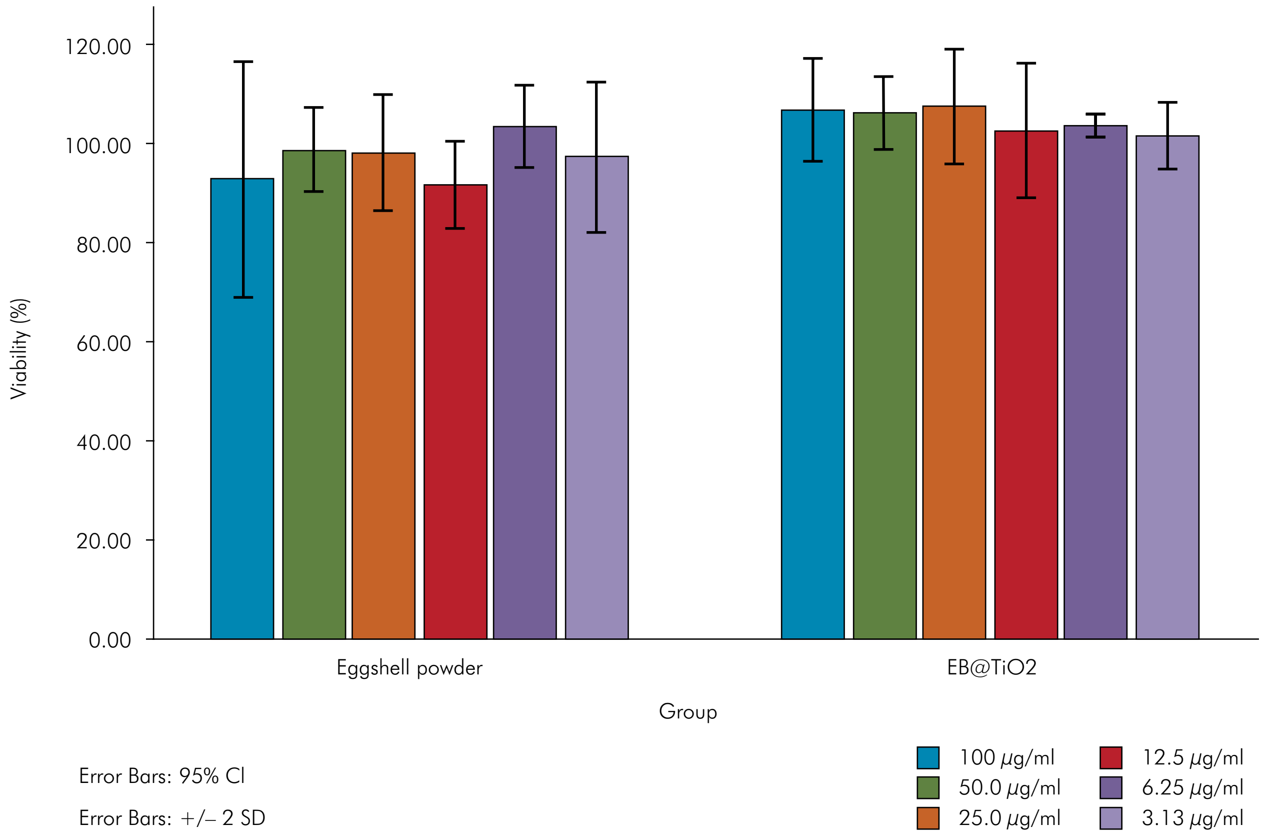

Figure 3. Percentage cell viability.

Table 1. ANOVA test for the occluded dentine tubules after treatment.

Variable	n	Mean	Std. Deviation	p-value
No treatment	4	-	-	0.000*
Eggshell powder	4	56.5	6.03	
EB@TiO_2	4	83.25	12.47	
Sensodyne	4	25.0	8.04	

Table 2. Bonferroni multiple comparison test.

Polishing material	Bonferroni	
	p-value	Sig.
EB@TiO2 Eggshell power	0.008	Significant
EB@TiO2 Sensodyne	0.000	Significant
Eggshell powder Sensodyne	0.003	Significant

for the specimens treated with eggshell powder (C2) and Sensodyne (C4), the images of the specimen treated EB@TiO_2 (C3) suggest more resistance to acidic challenge.

Discussion

It is well documented in literature that tooth erosion attributed to high consumption of citric acid containing soft drinks is increasingly seen as a public health concern, thus contributing to DH.[18] The potential strategy for management of DH is to effectively occlude the dentine tubules. The purpose of this study was to synthesized and evaluate the effectiveness of a ball-milled synthesized EB@TiO_2 to occlude open dentine tubules. As advocated by several authors,[19,20,21] bovine teeth were used in this study as a substitute for human teeth in the *in vitro* experiment. Interestingly, it is worth mentioning that the radicular dentine morphology of human and bovine primary teeth and root are similar in terms of the diameter of the dentine tubules.[22]

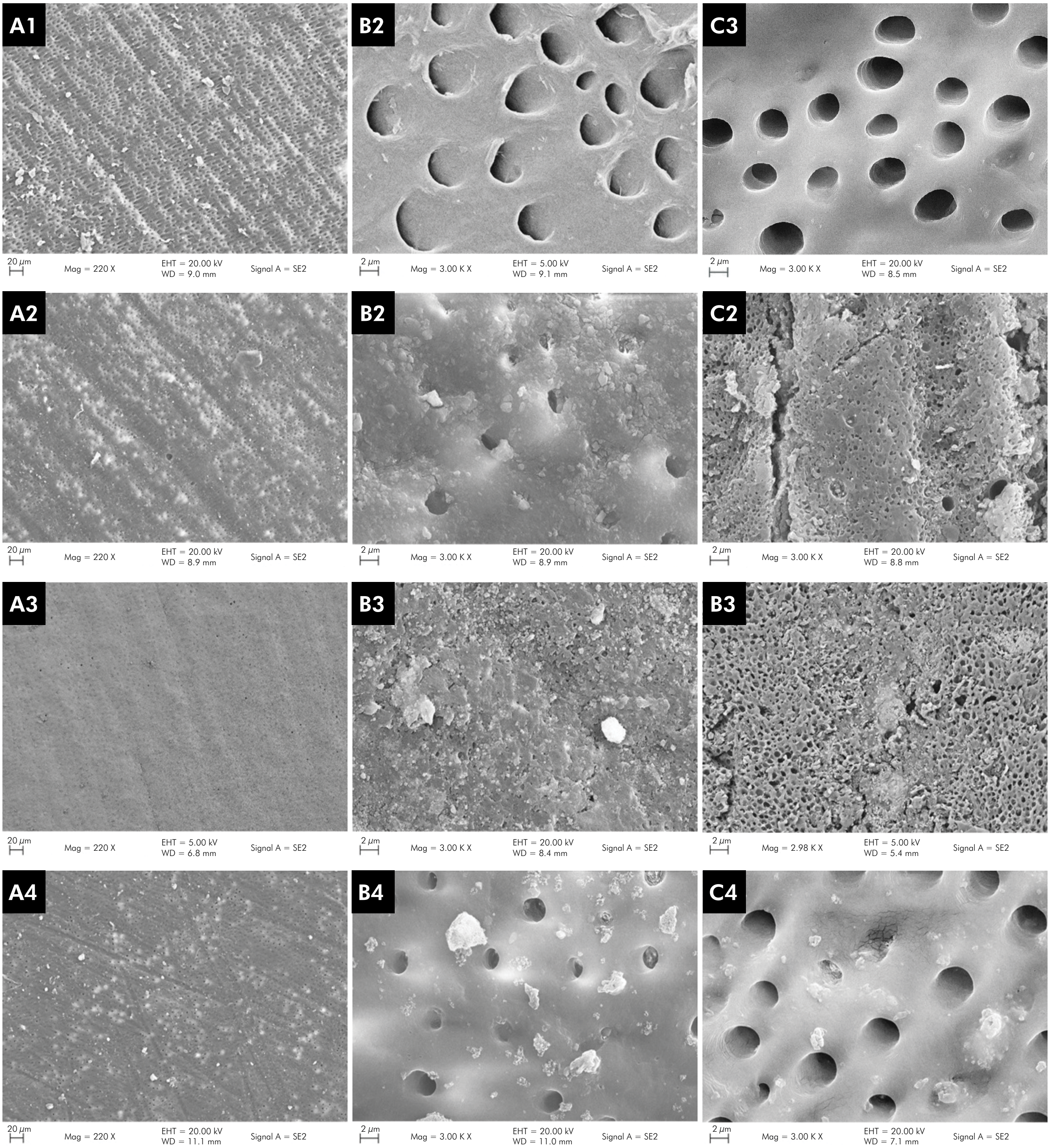

Figure 4. Representative SEM micrograph for dentine surface after treatment with different groups for 30min (A, x220; B x3000; Cx3000 Original magnification). (A1-B1) No treatment group; (A2-B2) eggshell powder treated group (A3-B3) EB@TiO_2 treated group; (A4-B4) Sensodyne treated group. (Agitated for 3hours). (C2) eggshell powder groups (C3) EB@TiO_2 treated group; (C4) Sensodyne groups. (post-exposure to 2wt. % citric acid solution for 5min).

Moreover, unlike human teeth, the bovine teeth with the more uniform composition are easy to obtain in large quantities with a relatively large flat surface and free of caries lesions and other defects.[21] SEM was used to analyze the surface of the dentine pre-and post- treatment in 2wt. % citric acid. The stated hypothesis was accepted base on the study finding, as the exposed dentine tubules treated with EB@TiO_2 demonstrate outstanding occlusion as well as acid resistant characteristics.

Overall, the occlusion of the dentine tubules observed in the samples treated with EB@TiO_2, eggshell powder, and Sensodyne were significantly different ($p < 0.001$). Given the statistical difference between the eggshell powder and EB@TiO_2 ($p < 0.008$), the observed differences could be attributed to the particle sizes of EB@TiO_2 (≤ 13 nm) and Eggshell powder (≤ 25 µm). As reported by Nakashima et al.,[9] nano-sized calcium carbonate containing materials can potentially remineralize damaged teeth due to their unique properties which facilitate the attachment on the oral surface. Owing to this attachment, calcium ions are released into the oral fluid that occludes dentinal tubules.

Moreover, the occluding abilities of Sensodyne toothpaste were found to be significantly lower than both eggshell powder ($p < 0.003$) and EB@TiO_2 ($p < 0.001$), respectively. The highest (83.2 ± 12.4) occlusion per area were observed in the samples treated with EB@TiO_2 while the lowest (25 ± 8.04) was measured in the group treated with Sensodyne toothpaste. These differences may reflect on the composition and design of the toothpaste. According to Schiff et al.,[23] desensitizing toothpastes such as Sensodyne Colgate Sensitivity toothpaste are originally designed to deliver potassium ions that acts as a nerve depolarizing agents in the treatment of DH. Nevertheless, the mean number of occluded dentinal tubules measured in the Sensodyne treated group showed that the toothpaste could still potentially occlude dentinal tubules. Consistent with the early report of Pashley et al.,[24] the observed occlusion of some tubules in the samples treated with Sensodyne may be attributed to the silica composition in the toothpaste. This strongly supports the work of Wang et al.[25] that toothpastes containing abrasive materials such as calcium carbonate and silica had the ability to form a new smear layer on the surface of the dentine; thereby occluding the dentinal.

Furthermore, the acid resistant characteristics of the treated samples were evaluated post-treatment using 2wt.% citric acid. Notably, the results visibly showed that acid resistant characteristics of EB@TiO_2 (Figure 4 C2) were superior to that of eggshell powder (Figure 4 C3) and Sensodyne toothpaste (Figure 4 C4). The acid resistant of EB@TiO_2 may have been influenced by the deposition of TiO_2 on the calcite (eggshell) surface (Figure 1). This is in agreement with the report of Tao et al.[15] that titanium dioxide can improve the acid resistant properties of calcium carbonates. On the contrary, for Sensodyne toothpaste, Arnold et al.[10] reported that although certain toothpaste like Sensodyne may support dentinal occlusion; such occlusion are superficial and are dissolved with acids.

In reviewing literature related to the present study, the occlusion abilities and acid resistant characteristics of Nano/micro fluorhyhydroxyapatite crystals,[26] nanohydroxyapatite/mesoporous[11] silica composite and zinc oxide hydroxyapatite paste [27] have shown promising results in the treatment of DH. Unlike the aforementioned materials, EB@TiO_2 bio composite had the advantage of being readily available since the bulk of the material is made from waste eggshell.

From an environmental sustainability and management perspective, and as argued by Onwubu et al.,[17] using eggshell waste material to treat DH will strengthen the economic benefits associated with using natural waste material, which is high on the global agenda for a greener environment. This argument is supported by Yazıcıoğlu and Ulukap [28] who pointed out that a low cost, affordable, feasible, and sustainable products need to be developed to repair and improved quality of life for patients who do not have to suffer toothache because of sensitivity. This suggests that EB@TiO_2 has the potential to be used as an oral care product in the management of DH. Importantly, the cytotoxicity result (Figure 3), suggests that EB@TiO_2 appeared to have little effect on the NIH 3T3 cell line. Further vivo research is, however, needed to fully and comprehensive characterize the cytotoxicity as well as the occluding potential of EB@TiO_2 bio composite. This is an uncharted area of research worth exploring.

Additionally, and despite the above outstanding occluding capabilities of EB@TiO_2, some limitation was noticed in the present study. The agitation procedure cannot be used to predict the rate and the number of days in an oral condition required to completely occlude the tubules. Hence future studies are planned to evaluate the occluding abilities of the composite using the brushing procedure stored in the presence or absence of saliva. These studies will help establish the EB@TiO_2 composite as an effective material to manage DH.

Conclusion

In conclusion, this study confirmed that modified EB@TiO_2 composite could effectively occlude dentine tubules. It was also demonstrated that the occlusion had depth and highly effective in acidic environment.

Acknowledgment

The authors acknowledged the financial support from the National Research Foundation of South Africa (No. 104824).

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Manuscript I

Onwubu, S.C., Mdluli, P.S., Singh, S., Makgobole, M. and Bharuth, V. 2019. Evaluation of the occluding characteristics of nano eggshell/titanium dioxide with or without saliva. European Journal of Dentistry **(Accepted for publication)**

Type of article: Original Article

Title of the article: Evaluation of the occluding characteristics of nanosized eggshell/titanium dioxide with or without saliva

Running title: Remineralization capabilities of EB@TiO_2 with or without saliva

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ABSTRACT

Objectives: The study reports on the effectiveness of a ball-milled nanosized eggshell titanium dioxide composite (EB@TiO_2) for DH management in comparison with commercial desensitizing paste with and without saliva.

Methods and Materials: Forty-nine dentine specimens were prepared from extracted bovine anterior teeth. Twenty-one of the specimens were brushed with 3 desensitizing toothpaste for seven days, namely: Group 1 EB@TiO_2, Group 2; Colgate Pro-relief; and Group 3: Sensodyne repair (n=7). Twenty four of the specimens were brushed with the toothpaste for seven days and stored in artificial saliva (control) after brushing. Each specimen were subsequently post-treated in citric acid solution to test their stability in acidic condition. Field Scanning Electron Microscope was used to evaluate the effectiveness of the dentine tubules occlusion. The biocompatibility of the composite was tested using BHK21 cell line.

Statistical Analysis Used: One way analysis of variance was used to analyze the % occluded area ratio values for all specimens (α=.05). Independent t-test was further used to evaluate the occlusion differences with saliva and without saliva.

Results and Conclusions: The number of dentine tubules decreases significantly after seven days of brushing. Overall, the occlusion observe for EB@TiO_2 were significantly better than for Colgate Pro-relief and Sensodyne repair ($P<0.05$). BHK21 assay suggests that composite showed no significant effect on the BHK21 cell line. This study demonstrated that the composite effectively occlude open dentine tubules within seven days of brushing.

Key-words: Dentine hypersensitivity, Desensitizing agents, remineralization,

Introduction:

Globalization has created a rapid change in the diets and lifestyles of millions of people worldwide. In South Africa for example, the reintroduction of the country to the global economy post-apartheid in 1994 has witnessed the proliferation of foreign goods and a rapidly changing food environment [1]. Concerning, and in the context of oral health care, this changes has brought about the increase in consumption of energy drinks, and acidic beverages; which is reportedly linked to the incidence of dental diseases such as dental caries and erosion of the enamel surface.[2] More worrisome is that the excessive demineralization of the tooth surface due to erosion has been reported to initiate the onset of dentine hypersensitivity (DH).[3, 4]

According to the Canadian Advisory Board on Dentine Hypersensitivity[5], DH is characterized by distinctive short, sharp pain arising from exposed dentinal tubules particularly in response to external stimuli that are typically thermal, evaporative, tactile, electrical, osmotic or chemical changes which cannot be ascribed to any other form of dental defects or pathology. As reported in the literature, DH is one of the most clinically encountered problems in dentistry affecting between 10-30% of people worldwide.[6] Although there have been conflicting reports on the exact prevalence of DH, nevertheless, the most common age range in which DH is frequently experienced is given as 20-50 years, with female patients predominantly affected.[7, 8] Moreover, and as Schiff *et al.*[9] points out, DH has a negative consequence on the quality of life for dental patient as they are less complaint with oral hygiene recommendation; thus posing a challenge for oral health care providers to manage.

Many theories have been reportedly proposed to explain the mechanism of DH. However, the hydrodynamic theory expanded upon by Brannstrom is now accepted by the dental community as the most likely mechanism for DH occurrence.[10] Consequently, approaches to control the hydrodynamic mechanism have resulted in the development of two class of products namely: (1) occluding products or agents that reduce fluid flow within the dentine tubules, and (2) agents or products that interfere with the transmission of nerve impulses.[9, 11] At the first line of at-home therapy in the management of DH, the use of occluding agents is often recommended for effective treatment.[12]

Several different occluding agents such as potassium oxalates[13], sodium fluoride and sodium monofluorophosphate[10], strontium salt[14], amorphous calcium phosphate containing casein phosphopeptide[15] and calcium glycerophosphate[16] have been widely utilized in desensitizing paste for their dentine tubule occluding capabilities. Still, the effectiveness of the aforementioned occluding agents will depend on the flow of saliva. Moreover, due to the chemical composition of saliva, it can play a critical role in naturally reducing DH [11, 17]. Kleinberg [17] revealed that saliva could reduce DH by supplying and carrying calcium and phosphate ions into open dentin tubules; which gradually bring about tubule blocking and by forming a surface protective layer consisting of precipitated aggregates of a combination of salivary glycoproteins with calcium phosphate. In some patients, however, particularly those with conditions of hyposalivation and xerostomia, the flow of saliva is limited; which could further increase the risks of caries and tooth demineralization; thereby exacerbating DH [18].

In an attempt to address the above concern, Kleinberg in 2012 at the State University of New York-Stony Brook, patented a novel occluding agents based on the understanding of the role that saliva plays n naturally reducing DH. This new technology comprises arginine (an amino acid with a pH 6.5-7.5), bicarbonate, pH buffer, and calcium carbonate [17]. The said technology is marketing under the brand name Colgate Pro-Argin [9]. It is reported that Pro-Argin technology function by occluding dentinal tubules using arginine to bind to the negatively charged dentin surface, which subsequently attracts a calcium-rich layer from the saliva to infiltrate and block the dentinal tubules [18]. However, its effectiveness in a highly acidic environment has been reported to be ineffective [19], thus leading to the reopening of the dentine tubules. Given the above drawbacks, a new occluding material based on the modification of eggshell powder and titanium dioxide was recently proposed for the management of DH [20].

Importantly, eggshells have been predicted to be the future of remineralization owing to its high bioavailability of calcium [21, 22]. Equally, it has been suggested in the literature that nano-sized titanium dioxide and dental abrasive agents can be used together in occluding open dentine tubules [23]. In a recent report, the authors demonstrated that modifying eggshell powder with titanium dioxide (EB@TiO_2) significantly improved it's acidic resistant to erosive acids [24]. This present study, therefore, aimed to evaluate the occluding characteristic of EB@TiO_2 in reducing DH compared with commercial toothpaste containing Pro-Argin (Colgate Pro-relief) and NovaMin (Sensodyne repair) with or without saliva. The formulated hypothesis tested was: EB@TiO_2 significantly occlude the open dentine tubules with or without saliva.

Materials and Methods:

Two commercially available toothpaste namely: Sensodyne repair (GlaxoSmithKline, UK) and Colgate Pro-relief (Colgate-Palmolive, Poland) were used as the test desensitizing paste. Titanium dioxide (Anatase; CAS No: 13463677) was purchase from Sigma-Aldrich (Germany), whilst the citric acid used was supplied by Merck (South Africa).

Preparation of Eggshell-Titanium dioxide composite (EB@TiO_2)

Eggshell and titanium dioxide composite was prepared in accordance with the method reported in literature [20]. An extensive details of the surface morphology, particle sizes and phase of the prepared EB@TiO_2 can be found in other reported papers [20, 25-26].

Preparation of artificial saliva

Artificial saliva were prepared following the method reported by Saporeti *et al.* [27] with a slight modification. As specified in Table 1, the listed chemical were prepared in IL of volumetric flask using deionized water. The pH of the prepared saliva was given as 6.5.

Table 1: Composition of the prepared artificial saliva (mg/l)

Chemicals	Concentration (mg/L)	Mass (g)
$NaH_2PO_3H_2O$	780	0.078
NaCl	500	0.05
KCl	500	0.05
$CaCl_2H_2O$	795	0.0795
NaS_9H_2O	5	0.0005
$(NH_4)_2SO_4$	300	0.03
Citric Acid	5	0.0005
$NaHCO_3$	100	0.01
Urea	1000	0.1

Preparation of Dentine Tooth specimens

Forty-nine anterior teeth extracted from bovine were collected from an abattoir, South Africa. Disinfecting and cleaning of the teeth followed by immersing in 10% chloroxylenol solution. With the aid of a diamond saw operating at a minimal speed, and cooled with water, the teeth were sectioned below the enamel-dentinal to prepare a dentine specimens having a dimension of 5mm x 5mm x 1mm. A silicon carbide paper with particle size of 600grits were further used to wet ground the specimens for 60 seconds. Thereafter, the specimens were embedded in a resin (AMT composite, South Africa). The specimens were then soaked in a solution containing 4% wt citric acid for 2 min to open up the tubules. As described in Table 2, the specimens were randomly assigned in different experimental groups.

Each specimen from the respective groups were brushed twice daily (morning and evening) with a toothbrush powered with 1.5v alkaline battery (Oralwise, China) for 1min and allowed to dry for 30s before rinsing with deionized water. Brushing was performed at room temperature using 100mg of each respective toothpaste. The slurry of EB@TiO_2 was prepared by mixing 100mg of the powder/200 µL of deionized water. After each brushing protocol, the specimens were immersed in saliva or without saliva as described in Table 2. At the end of the seven-day brushing, the treated specimens were exposed to 4% wt. citric acid solution for 2 min to determine the resistance to acidic challenge, and subsequently rinsed in deionized before blot drying.

Table 2: The distribution of specimens according to the experimental group

Sample groups	Treatment condition		Brushing days	Total
	Without saliva	With Saliva		
Artificial saliva	-	7	Twice daily (for seven days)	7
EB@TiO2	7	7		14
Colgate Pro relief	7	7		14
Sensodyne repair	7	7		14
Total	21	28		49

Surface examination of the treated specimens

Field Scanning Electron Microscope (FESEM; Carl Zeiss) was used to examine the treated specimens after each day of brushing from each respective groups. The instrument was operated in controlled environment and scan at 20 kV. Prior to FESEM observation, the specimens were dehydrated, sputter coated with electric conductive gold film. Using the captured image of 1500 magnification, ImageJ software (National Institute of Health USA, http://imagej.nih.gov./ij) was used to compute the occluded tubules ratios by dividing the area of the occluded tubules by the total tubules area (n=7). The % occluded area ratio were counted and used for statistical evaluation.

Biocompatibility test

A cytotoxicity assay was carried out on the prepared EB@TiO_2 to evaluate its biocompatibility. Before culturing, the sample was dispersed in a solvent (Dimethyl Sulfoxide). The BHK21 hamster kidney cells were grown in the laboratory following the process of culturing normal tissues [20]. The cell viability were then evaluated using MTS assay. Auranofin was used as a negative control. All analyses were tested in duplicate and carried out across 2 plates (n=6).

Statistical analysis

1-way analysis of variance (ANOVA) was used to analyze the mean occluded area ratio within the different groups, followed by a Bonferroni test (α=.05). In addition, the independent t-test were used to compare the mean occluded area ratio observe for the specimens treated with saliva and without saliva (α=.05). All analysis were performed using statistical software (IBM SPSS Statistics v24; IBM Corp).

Results:

Dentine specimens treated in seven days (without saliva)

Table 3 depicts the mean, standard deviation, standard error, and ANOVA results. The total mean % ratio of the tubules occluded area for the dentine specimens treated with EB@TiO_2, Colgate Pro-relief, and Sensodyne repair was statistically different (P<.001).

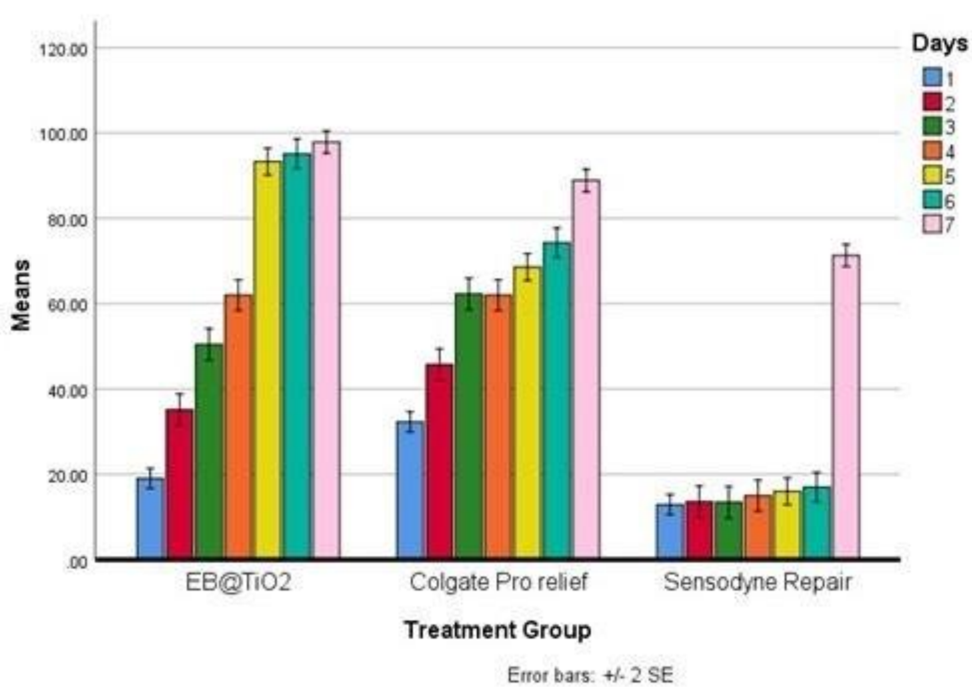
Notably, and after seven days of brushing, the EB@TiO_2 group had the highest % mean occluded area (64.7 ±1.3 %), while the Sensodyne repair treated group had the lowest % mean occluded area (22.7 ±4.8 %). The Bonferroni correction results re given in Table 3. The % tubules occluded for the test group (EB@TiO_2) were statistically higher when compared against the Colgate Pro-relief group (*P*<.05), and the Sensodyne repair group (*P*<.001). Equally, the % occluded area measured for the Colgate Pro-relief were significantly higher than that observed for Sensodyne repair (*P*<.001).

Table 3 ANOVA test Comparison of the occluded area (without saliva)

Treatment Group	N	Mean +SD	Std. Error	95% Confidence Interval		P	**Post hoc Bonferroni test**
				Lower Bound	Upper Bound		P
EB@TiO2	7	64.7± 1.3	.655	63.318	66.070	0.000	0.028[1,2]
Colgate Pro-relief	7	62.0± 3.2	.655	60.624	63.376		0.000[1,3]
Sensodyne Repair	7	22.7± 4.8	.655	21.358	24.111		0.000[2,3]

Superscript numbers indicate significant differences between the sample groups (ANOVA, *P* < 0.05).

Figure 1 illustrates the differences in the % tubules occluded per day with the 3 desensitizing paste materials (EB@TiO$_2$, Colgate Pro-relief, and Sensodyne). It was observed that as the brushing days increase, so where the % of the tubules occluded. At



the end of seven days brushing, EB@TiO_2 had the highest occluded area (97.8±1.3%) followed by Colgate Pro-relief (88.9 ±3.2%), and lastly Sensodyne repair (71.3 ±4.9%).

Figure 1: Differences in mean tubules occluded of dentine specimens treated with EB@TiO_2 Colgate Pro-relief, and Sensodyne repair desensitizing paste materials after 2 minutes of brushing without saliva immersion (seven day brushing test (n=7)).

Specimens Post treated in 4% citric acid (without saliva)

The Paired sample test, mean, and standard deviation results for the dentine specimen's pre- and post- acidic challenge are given in Table 4. There was no significant different found in the EB@TiO_2 group pre- and post-acidic treatment (P>0.05). It was observed that the occluded area ratio for the EB@TiO_2 group post-acid challenge (97±1.2), were comparable with those measured pre-acidic exposures (97.9±1.3). In contrast, both the Colgate Pro-relief and Sensodyne repair treated group showed differences (P<0.001).

Table 4: Paired sample test comparison of occluded area ratio pre-and post-acidic treatment

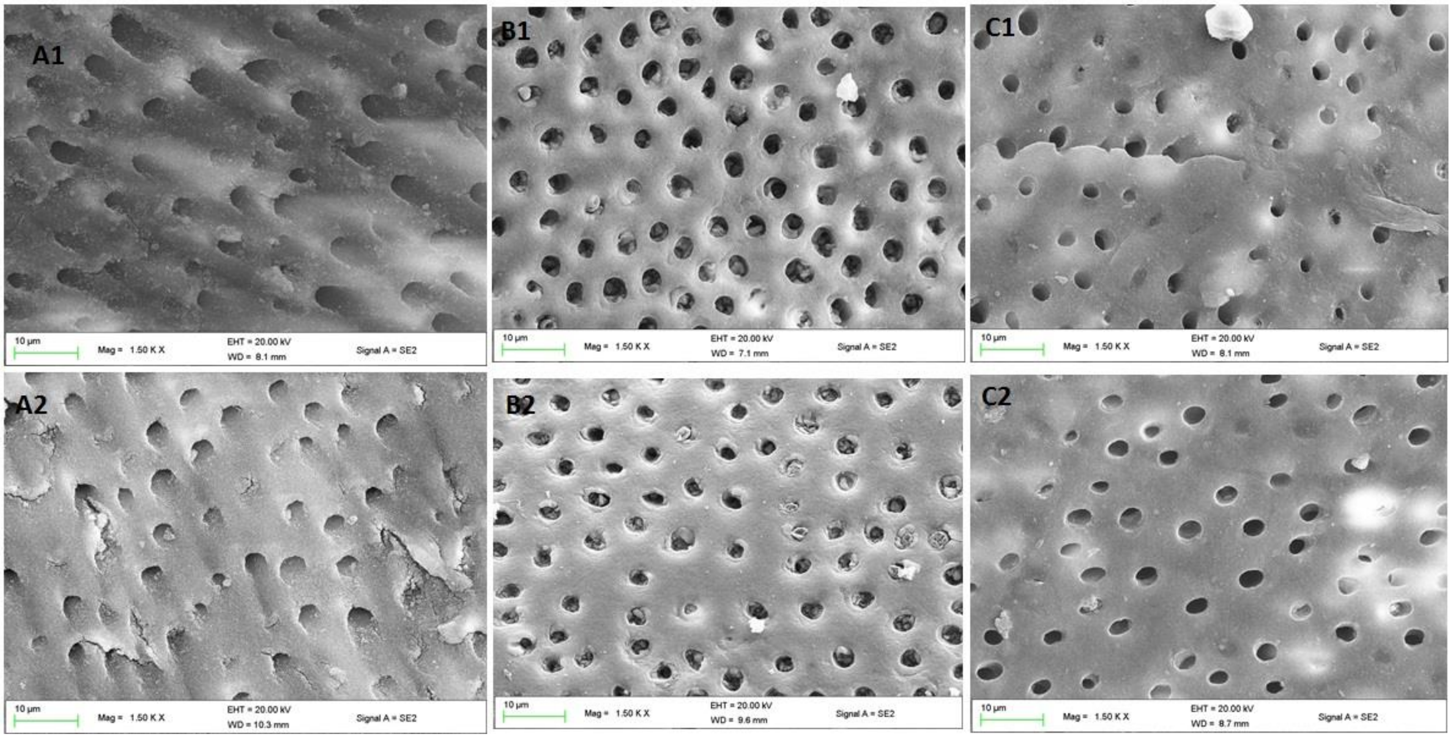
Treatment group	Occluded area (%)		*P*
	Pre acidic challenge (Mean+SD)	Post acidic challenge (Mean+SD)	
EB@TiO2	97.9±1.3	97.1±1.2	0.318
Colgate Pro-relief	88.9±3.2	33.9±4.1	0.000
Sensodyne repair	71.3±4.9	9.3±2.4	0.000

Field Scanning Electron Microscope observation of the occluded tubules (without saliva)

The FESEM image of the occluded dentine tubules for dentine specimens treated without storing in artificial saliva after seven days brushing test is reflected in Figure 2. In day 1 (Figure 2 B1) and 2 (Figure 2 B2), the group treated with Colgate Pro-relief appear to show more evidence of tubule occlusion. At a glance, it can be observed that there was a narrowing of the tubule length in the Colgate Pro-relief group. In contrast, the group treated with EB@TiO_2 and Sensodyne repair shows no visible changes in their tubules.

In day 3 (Figure A3 and B3) and 4 (Figure A4 and B4), although the occlusion of the exposed tubule was practically more visible in the specimens treated with Colgate Pro-relief, the EB@TiO_2 group appear to be showing evidence of tubule remineralization. However, there were no visible changes in the group treated with Sensodyne repair (Figure C3 and C4). In day 5 (Figure A5), 6 (Figure A6), and 7 (Figure A7), for the specimens treated with EB@TiO_2, there was complete remineralization or sealing of the tubules whilst some exposed tubules were still visible in the group treated with Colgate Pro-relief. On the contrary, the group treated with Sensodyne repair had no visible changes in the occlusion of the tubules in day 5, and 6, but, however, shows evidence of remineralization in day 7.

Further to the above, Figure 2 (A-C8) revealed the dissimilarity post-treatment in citric acid solution (4wt %). Nonetheless, the specimen treated EB@TiO_2 (Figure A8) showed superior acid resistance with no visible differences pre-and post-acidic challenge when compared against the specimens treated with Colgate Pro-relief (Figure B8) and Sensodyne repair (Figure C8), respectively.



A1
10 µm Mag = 1.50 K X EHT = 20.00 kV WD = 8.1 mm Signal A = SE2
B1
10 µm Mag = 1.50 K X EHT = 20.00 kV WD = 7.1 mm Signal A = SE2
C1
10 µm Mag = 1.50 K X EHT = 20.00 kV WD = 8.1 mm Signal A = SE2
A2
10 µm Mag = 1.50 K X EHT = 20.00 kV WD = 10.3 mm Signal A = SE2
B2
10 µm Mag = 1.50 K X EHT = 20.00 kV WD = 9.6 mm Signal A = SE2
C2
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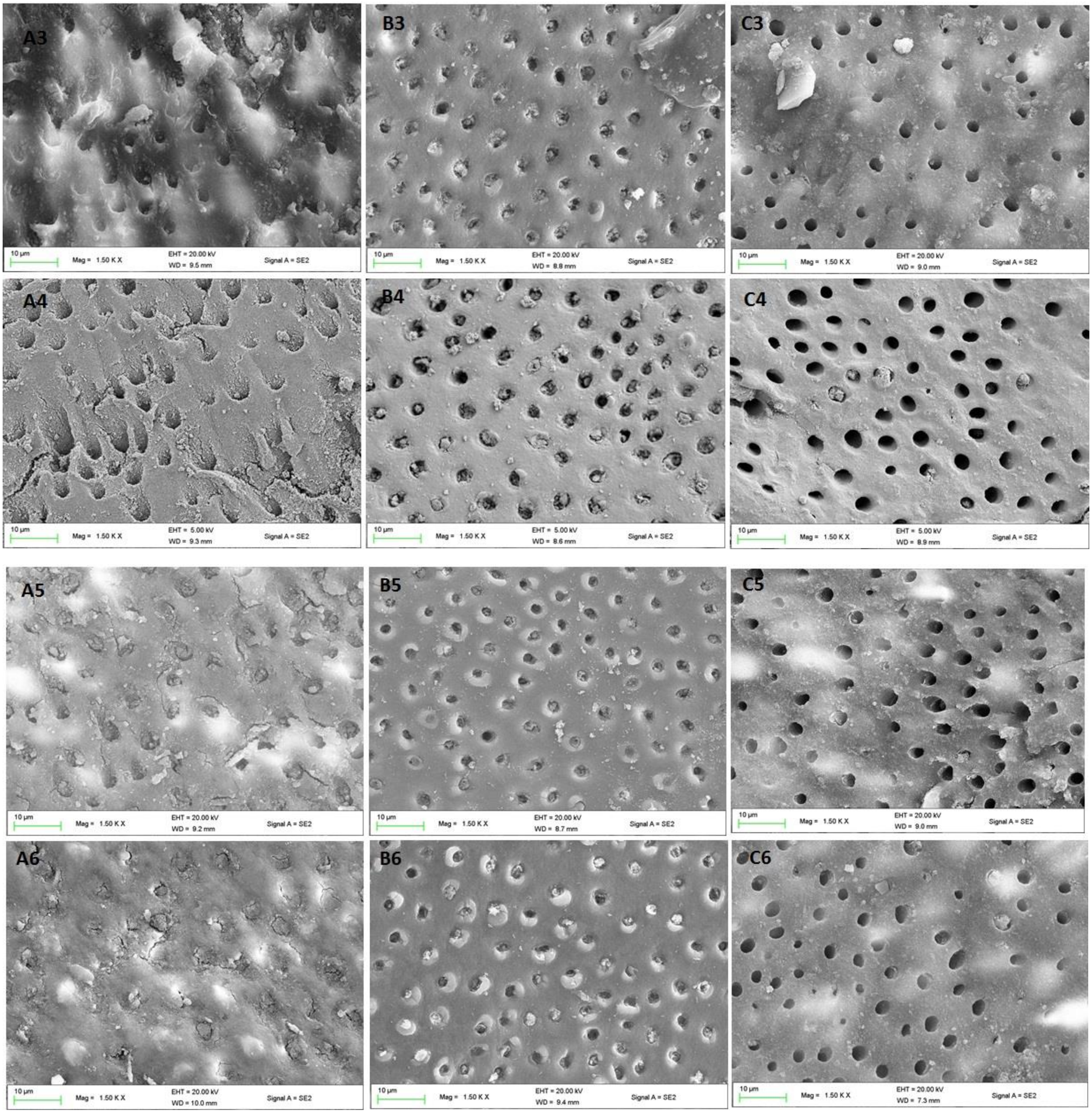
A3
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 9.5 mm
Signal A = SE2
B3
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 8.8 mm
Signal A = SE2
C3
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 9.0 mm
Signal A = SE2
A4
10 µm
Mag = 1.50 K X
EHT = 5.00 kV
WD = 9.3 mm
Signal A = SE2
B4
10 µm
Mag = 1.50 K X
EHT = 5.00 kV
WD = 8.6 mm
Signal A = SE2
C4
10 µm
Mag = 1.50 K X
EHT = 5.00 kV
WD = 8.9 mm
Signal A = SE2
A5
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 9.2 mm
Signal A = SE2
B5
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 8.7 mm
Signal A = SE2
C5
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 9.0 mm
Signal A = SE2
A6
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE2
B6
10 µm
Mag = 1.50 K X
EHT = 20.00 kV
WD = 9.4 mm
Signal A = SE2
C6
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Mag = 1.50 K X
EHT = 20.00 kV
WD = 7.3 mm
Signal A = SE2

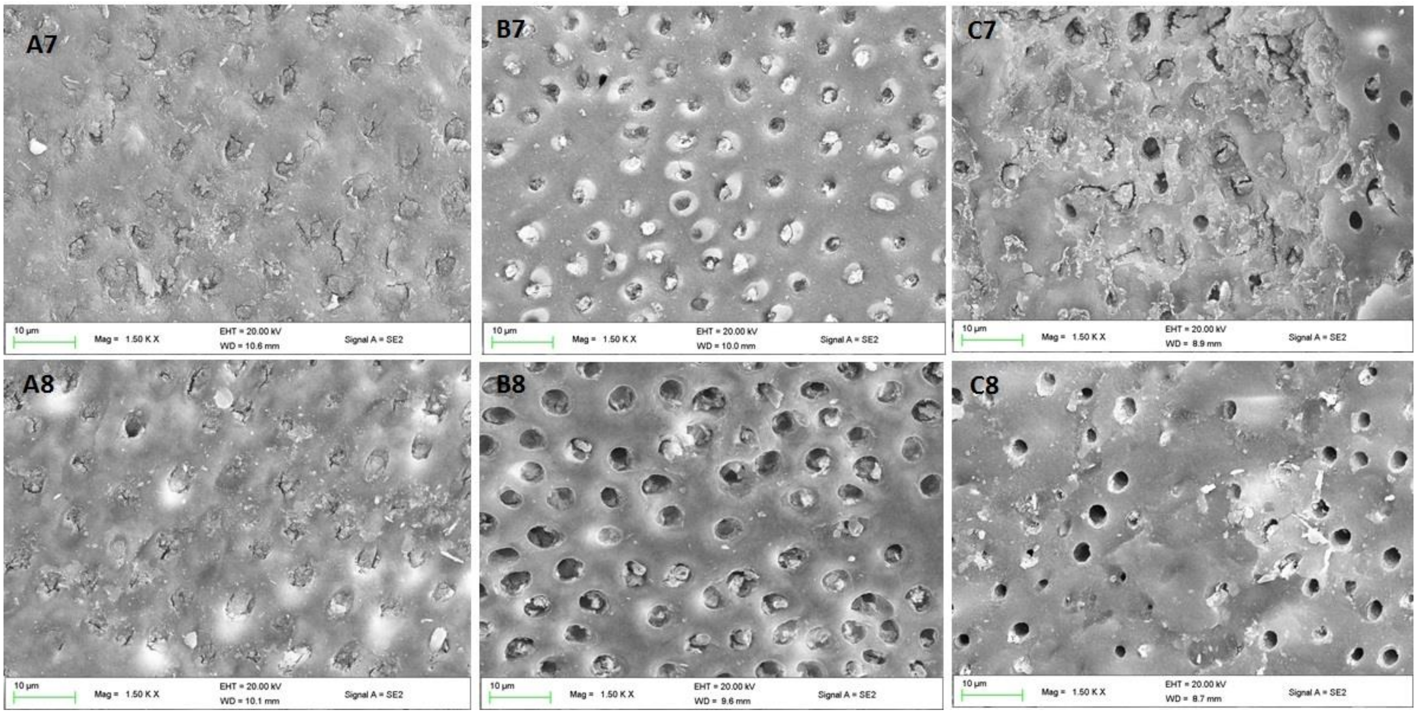


Figure 2: Representative FESEM micrograph for the dentine surface after brushing for seven days without saliva immersion using (A) EB@TiO_2; (B) Colgate Pro-Relief; (C) Sensodyne repair (1-7 represents number of each day of brushing with the respective desensitizing paste, and 8 represent post acidic exposure)

Dentine specimens treated in seven days (with saliva)

The mean, standard error, standard deviation, and ANOVA results for the dentine specimens stored in artificial saliva after brushing treatment are shown in Table 5. The total mean % ratio of the tubules occluded area for the dentine specimens stored in artificial saliva alone, treated with EB@TiO_2, Colgate Pro-relief, and Sensodyne repair was statistically different (P<.001).

It was found that the % occluded mean measured for the EB@TiO_2 group was the highest (72.0 ±1.0 %), while the specimens stored in artificial saliva alone without treatment had the lowest % mean occluded area (7.3 ±2.3 %). The Bonferroni correction results are shown in Table 5. The EB@TiO_2 group mean % occluded area was statistically better when compared against the Colgate Pro-relief group (P<.001), and the Sensodyne repair group (*P*<.001). More so, the % occluded area measured for the Sensodyne repair was significantly higher than that observed for Colgate Pro-relief (*P*<.001). All the treatment groups show a significant improvement in the occluding the tubules when compared against the samples stored in artificial saliva alone (*P*<.001).

Table 5: ANOVA test Comparison of the occluded area (samples stored in artificial saliva)

Treatment group	N	Mean + SD	Std. Error	95% Confidence Interval		P	**Post hoc Bonferroni test**
				Lower Bound	Upper Bound		P
Artificial saliva		7.3±2.3	.636	5.953	8.578	0.000	$0.000^{1,2,3,4,5}$
EB@TiO2		72.0±1.0	.636	70.708	73.333		$0.000^{2,3}$
Colgate Pro-relief		34.3±8.6	.636	33.034	35.659		$0.000^{3,4}$
Sensodyne repair		50.3±3.0	.636	49.014	51.639		$0.000^{2,4}$

Superscript numbers indicate significant differences between the sample groups (ANOVA, *P* < 0.001).

Figure 3 illustrates the differences in the % tubules occluded per day with the 3 desensitizing paste materials (EB@TiO_2, Colgate Pro-relief, and Sensodyne) and artificial saliva. It was observed that as the brushing days increase, so where the % of the tubules occluded. At the end of seven days brushing, EB@TiO_2 had the highest occluded area (99.3±1.0%) followed by Sensodyne repair (90.4 ±3.0%), and then Colgate Pro-relief (80.1±8.6%). Unsurprisingly, the specimens stored in saliva alone without treatment showed no significant changes in the occluded area (10.9 ±2.3%).

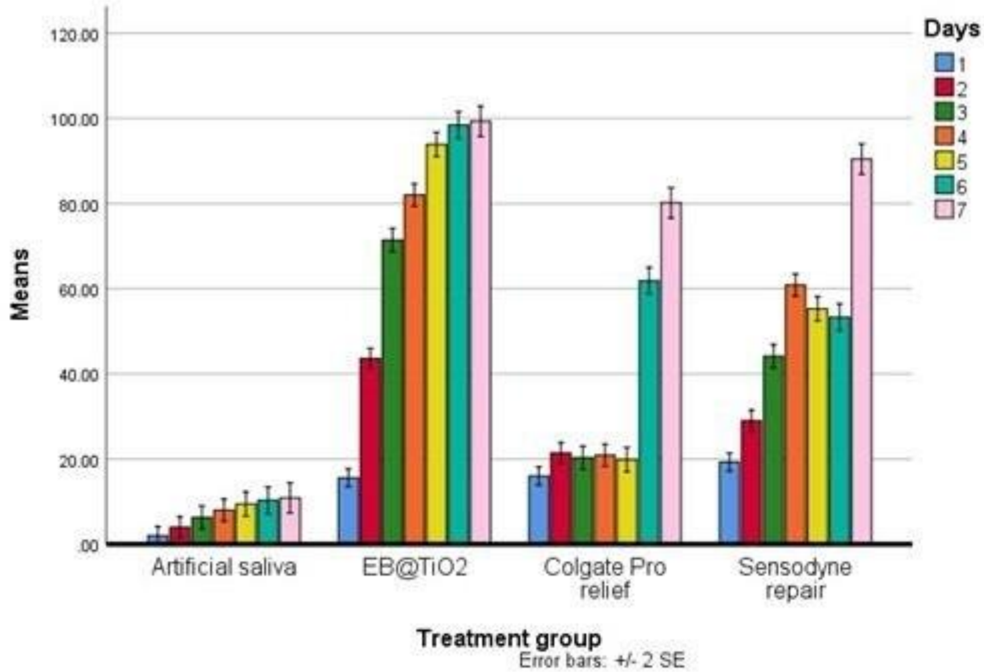


Figure 3: Differences in mean tubules occluded of dentine specimens treated with EB@TiO_2 Colgate Pro-relief, and Sensodyne repair desensitizing paste materials after 2 minutes of brushing and immersed in saliva (seven day brushing test (n=7)).

Specimens post-treated in 4% citric acid (with saliva)

Table 6 provides the paired sample test, mean, and standard deviation results for the dentine specimen's (stored in artificial saliva) pre- and post- acidic challenge. No statistical difference was found in the Sensodyne repair group pre- and post-acidic treatment ($P>0.05$). By contrast, there was a significant difference observed for the EB@TiO_2, Colgate Pro-relief, and specimens stored in artificial saliva alone ($P<0.001$). For the desensitizing paste, it was found Sensodyne repair achieved the highest acid-resistant stability while Colgate Pro-relief had the lowest.

Table 6: Paired sample test comparison of occluded area ratio pre-and post-acidic treatment (samples stored in artificial saliva)

Treatment group	Occluded area (%)		*P*
	Pre acidic challenge (Mean+SD)	Post acidic challenge (Mean+SD)	
Artificial saliva	10.9±2.3	5.7±1.8	0.001
EB@TiO2	99.3±1.0	85.0±3.8	0.000
Colgate Pro relief	80.1±8.6	9.0±2.2	0.000
Sensodyne repair	90.4±3.0	88.1±3.9	0.245

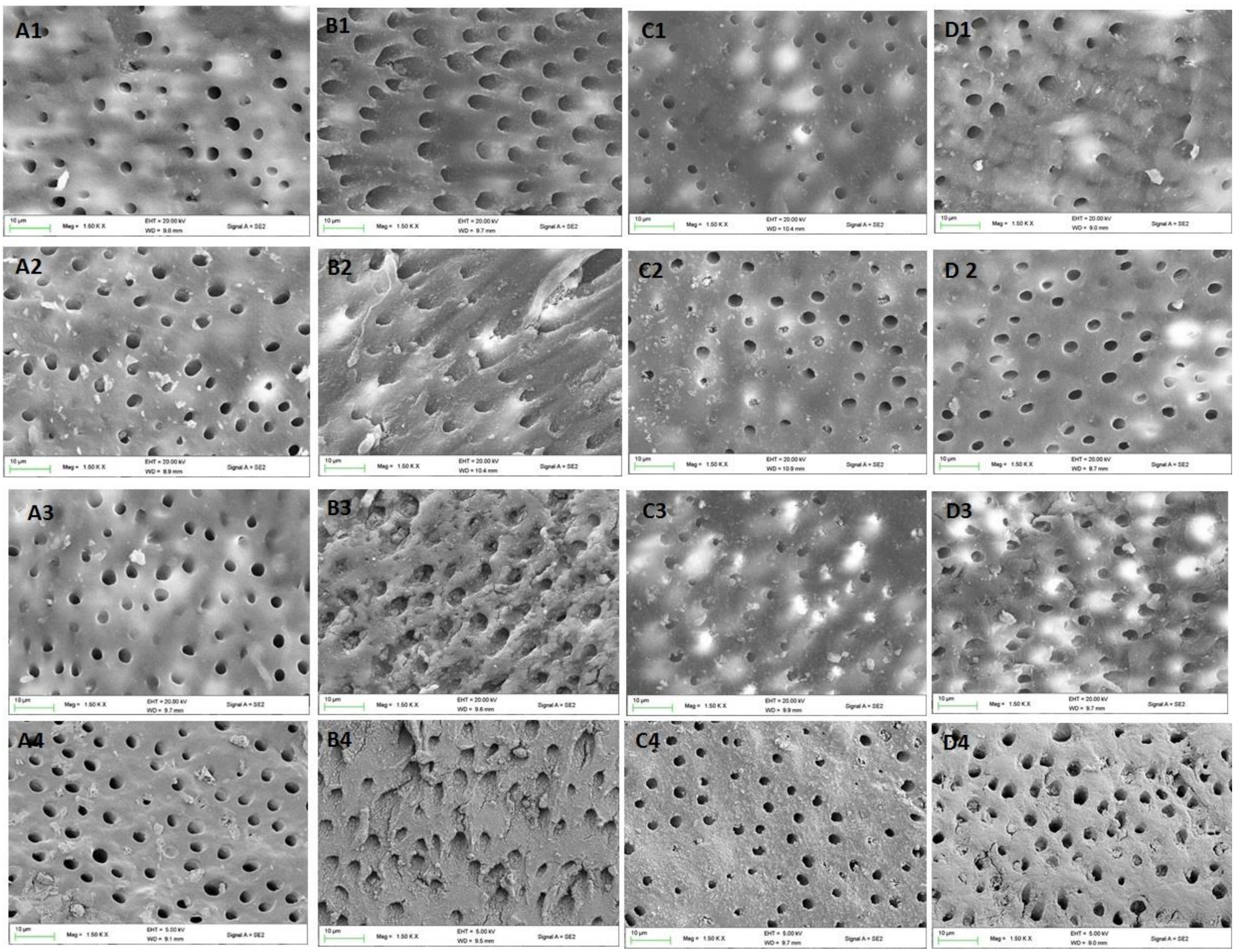
Field Scanning Electron Microscope observation of the occluded tubules (with saliva)

The FESEM images of the dentine specimens treated with EB-TiO_2, Colgate Pro-relief, and Sensodyne repair for seven days and storing in artificial saliva are shown in Figure 4. The observed images indicate the occlusion of EB-TiO_2 groups were different from other test groups (Artificial saliva, Colgate Pro-relief, and Sensodyne repair).

In day 1, for example, no noticeable change was observed in the specimens from all the groups. In day 2, 3, and 4, evidence of remineralization could be observed in the EB@TiO_2 treated group (Figure A1-A4). In contrast, no remarkable changes noticed in the other tested groups (Artificial saliva, Colgate Pro-relief, and Sensodyne repair). In day 5 (Figure A5), there was complete remineralization or sealing of the tubules in the group treated with EB@TiO_2 while the Sensodyne treated group was beginning to show evidence of occlusion. Similar trends as day 5 were noticeable in the group treated with EB@TiO_2 and Sensodyne repair at day 6. More so, the Colgate treated group at day 6 shows some visible occlusion in the tubules.

In day 7, both EB@TiO_2 (Figure A7) and Sensodyne repair (Figure C7) had comparable evidence of complete remineralization with Colgate Pro-relief (Figure B7) showing partial or complete occlusion of the tubules. Expectedly, the groups treated with artificial saliva had no evidence of occlusion (Figure D7).

Added the above, Figure 4 (A-D8) revealed dissimilarity post-treatment of the specimens in citric acid solution (4wt %). For both the EB@TiO_2 and Sensodyne treated group, for example, the occluded tubules appear to remain intact after an acidic challenge. On the contrary, the tubules in the Colgate Pro-relief (Figure 4 C8) treated specimens were visibly reopened post-acidic challenge.



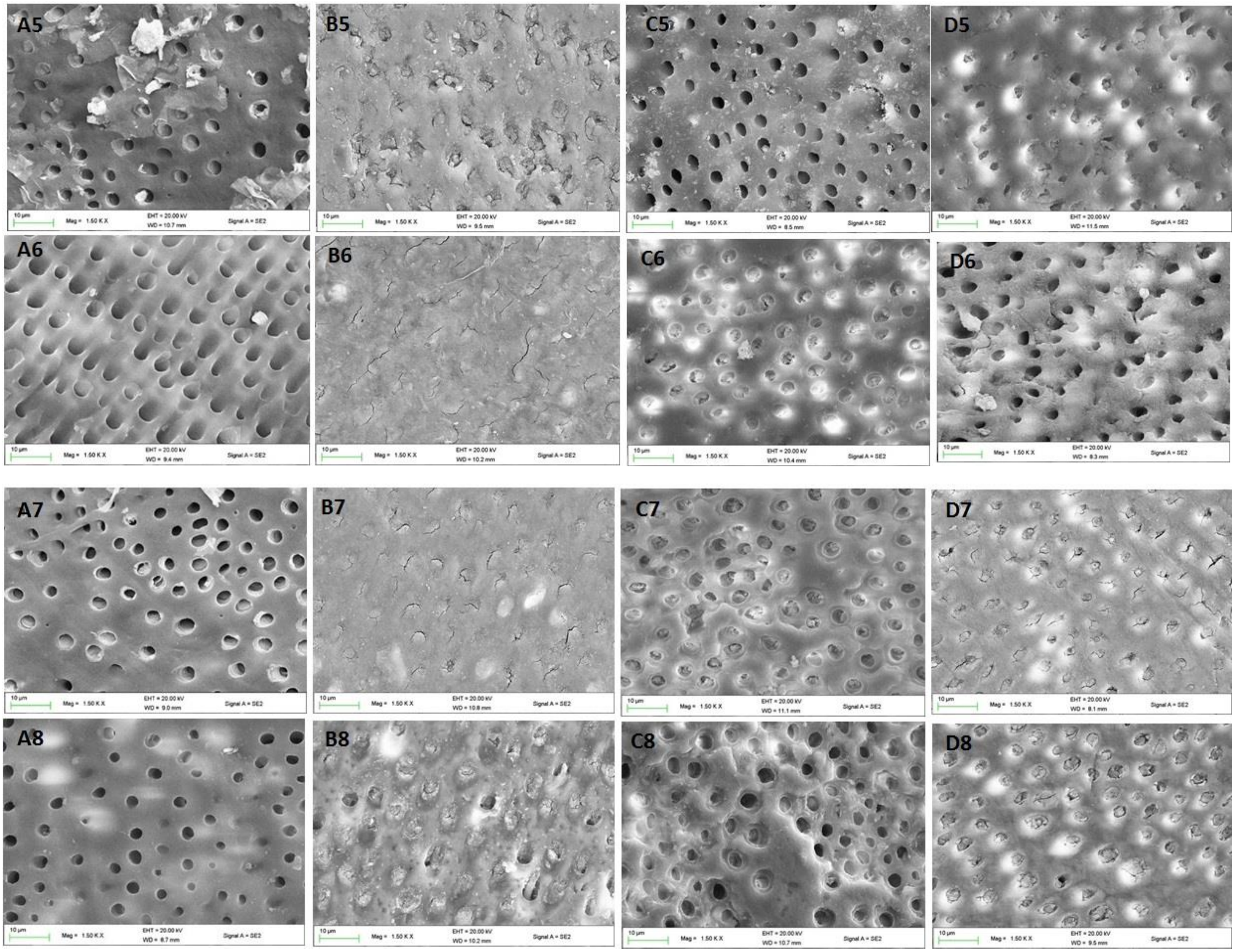


Figure 4: Representative FESEM micrograph for the dentine surface after brushing for seven days with saliva immersion using (A) Artificial saliva; (B) EB@TiO_2; (C) Colgate Pro-Relief; (D) Sensodyne repair (1-7 represents with the respective desensitizing paste, and 8 post acidic exposure)

Biocompatibility testing

The biocompatibility of $EB@TiO_2$ with the BHK21 cell line is shown in Figure 5. In comparison to the negative control, the $EB@TiO_2$ appear to show little effect on the BHK21 cell lines. However, there was 56% cell viability at 100 µg/ml.

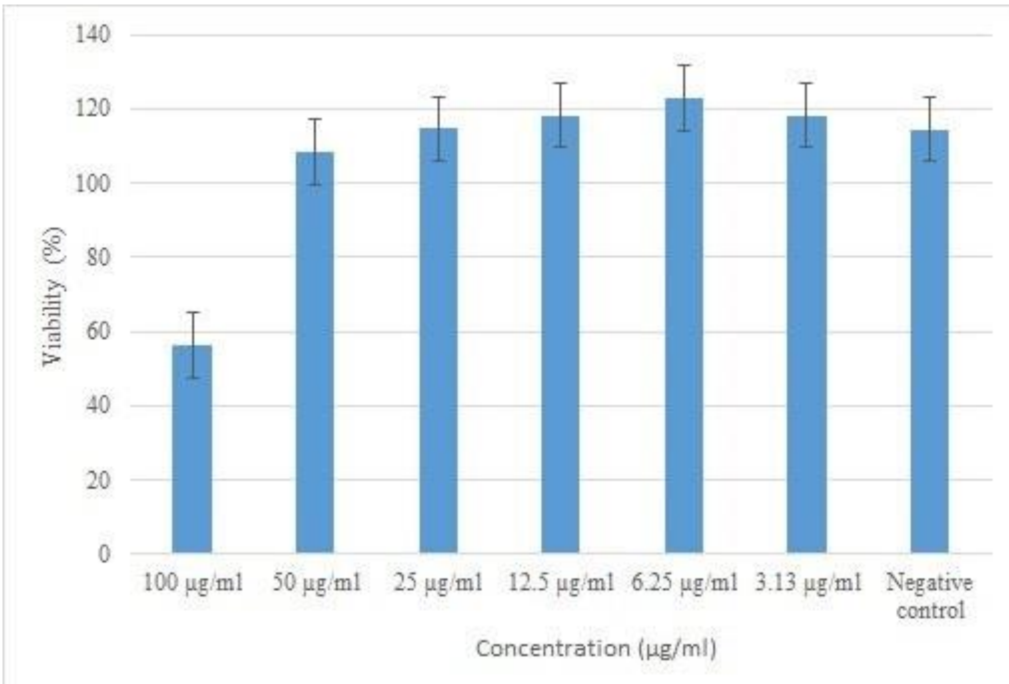


Figure 5: Percentage cell viability

Discussion:

Over the last decade, dentine hypersensitivity (DH) has been extensively researched owing to its widespread prevalence and noticeable painful oral health problem affecting many individuals [28]. The purpose of this study was to evaluate the effectiveness of a modified nanosized eggshell powder titanium dioxide composite ($EB@TiO_2$) in reducing DH. The composite was prepared through the mechanochemical activation method. Importantly, this method utilizes a mechanical energy to create structural changes as well as stimulate chemical reactions [29]. Consequently, it becomes possible to cause structural changes and particle size reduction in the EB@TiO_2 composite [25,26]. Conversely, the effectiveness of the prepared EB@TIO_2 in reducing DH was compared against Colgate Pro-relief and Sensodyne with or without immersion saliva. As suggested in the literature [30-32], their occluding capabilities were evaluated using the bovine model. The morphological changes of the dentine tubules pre-and post-acidic treatment were evaluated with FESEM. The EB@TiO_2 treated specimens showed good tubule occlusion that still remain effective in acidic condition for both samples treated with and without saliva. This lead to the acceptance of the study hypothesis.

With respect to time, in the specimens treated without saliva, Colgate Pro-relief showed instant occluding of the dentine tubules. This is consistent with clinical studies [33, 34] that Pro-Argin technology provides instant relief of DH. According to the mechanism proposed by Kleinberg [17], it can be suggested that the positively charged arginine in the Colgate Pro-relief is attracted to the negatively charged dentin surface where it helps attract and adhere calcium carbonate to the dentin surface; which in turn promote the occlusion of the tubules. Despite this, the overall dentine tubule occlusion observed in the samples treated with EB@TiO_2 were significantly better than Colgate Pro-relief ($P<0.05$) and Sensodyne repair ($P<0.001$), respectively. This differences could be attributed to the modification of the carbonate structure in eggshell with titanium dioxide [20]. According to Cutler [22], nanosized titanium dioxide together with abrasive materials facilitate the occluding of dentine tubules, thus contributing to reducing of DH. Added to this, the nanosized calcium carbonate materials have unique high surface energy–thus facilitating the attachment of calcium-rich ions on the oral tooth surface [35].

Furthermore, the occluding abilities of Sensodyne repair were found to be significantly lower than Colgate Pro-relief ($P<0.001$). The highest (64.7±1.3) occlusion per area were observed in the samples treated with EB@TiO_2 while the lowest (22.7±4.8) was measured in the group treated with Sensodyne repair. This difference may be related to the composition of the various test materials. Although studies [36, 37] have shown that calcium sodium phosphosilicate (NovaMin) constituent of the Sensodyne repair could obstruct dentine tubules to some extent, Yu *et al.* [38], however, argued that the Ca^{2+} and PO_4^{3-} are protected by glass particles which need to be trapped for the Ca^{2+} and PO_4^{3-} to be localized. The consequence of this is that there might be a delay in the action of the NovaMin to effectively promote the closing of dentine tubules [38]. Since the brushing test was carried out in seven days, without saliva, the inferior occluding characteristics observe for Sensodyne repair could be attributed to the absence of saliva to trap the Ca^{2+} and PO_4^{3-}.

On the other hand, for the specimens treated with saliva immersion, all the tested material demonstrated a significant occlusion difference when compared to the those found in saliva alone ($P<0.001$). While saliva is reported to facilitate remineralization by the deposition of calcium and phosphate [11, 17], the finding from this study suggests that the occlusion of specimens in saliva alone without desensitizing paste treatment were highly inferior. This may, however, be attributed to the treatment duration (Table 2). As reported in literature [10], the occluding capabilities of saliva occur gradually within a long time. In support of the role saliva plays in reducing DH, the dentine tubules occlusion observe for Sensodyne repair showed an outstanding occlusion when compared against the samples treated without saliva. Similar significant occluding abilities were measured for EB@TiO_2 treated with saliva immersion ($P<0.05$).

Contrary to the above, the dentine tubules occlusion observe for the samples treated with Colgate Pro-relief with saliva treatment were consistently inferior at each day of brushing to those measured for the samples treated without saliva ($P<0.001$) (Table 6). In contrast, other studies [9, 33] have alleged that the association of the arginine and calcium carbonate in situ provides an alkaline environment which encourages endogenous calcium and phosphate ions to deposit and occlude the dentin tubules. However, Yang *et al.* [39] found that Colgate Pro-relief showed no significant changes after treatment and immersion in artificial saliva for 14 days. The above author findings corroborate with the same observation found in our study.

Moreover, acid resistant stability is an important criterion for evaluating the efficiency of desensitizing paste in occluding dentine tubules [40]. This is more important as the oral cavity is often bombarded with citric acid that is highly common in the soft drinks and fruit juices found in our daily diets. In light of these, the effectiveness of the dentine occlusion observed with the different desensitizing paste was assessed post-treatment in a solution containing 4wt.% citric acid. The results observed for Colgate Pro-relief suggests that the product demonstrated an acid resistant to a certain extent. This can further be supported by the FESEM images that visibly showed that some of the closed dentine tubules were reopened after exposure to the citric acid solution (Figure 2 B8, and 4 C8). This, however, could be attributed to the solubility of calcium phosphates in an acidic environment [19].

In terms of the Sensodyne repair, the acid resistance effectiveness measured exhibit different behavior in the samples treated with and without saliva. In the group treated without saliva, nearly all the tubules were reopened after the exposure to citric acid (Figure 2 C8). Similar findings were observed by Yu *et al.* [38] were the deposits created by NovaMin on the dentine surface were almost completely removed by the citric acid solution. In contrast, the samples treated with saliva (Figure 4 D8), the Sensodyne repair demonstrated an outstanding acidic resistance characteristic ($P>0.05$). The difference observes for both sample treatment may be associated with the role the saliva plays. It can, therefore, assume that the occlusion for the samples treated with Sensodyne and immersed in saliva had depth and penetration thereby contributing to its acidic resistance.

As for the EB@TiO_2 group, the acidic resistant properties observe for the samples treated without saliva, pre and post citric acid exposure were comparable ($P>0.05$). However, slight differences were observed for the samples treated and immersed in saliva. It was found that after citric exposure, some of the obstructing tubules were reopened (Figure 4 B8). This notwithstanding, the FESEM images visibly validate that the acid resistant characteristics of EB@TiO_2 were superior to that of Colgate Pro-relief and to some extent Sensodyne repair. Consistent with the report by Tao *et al.* [41], the acid-resistant of EB@TiO_2 may have been influenced by the modification of eggshell with titanium dioxide. Further clinical research is, however, needed to substantiate the efficiency of EB@TiO_2 as biocomposite material for the management of DH.

Conclusion

In conclusion, and within the study limitation, the study established that the EB@TiO_2 composite successfully occludes open dentine tubules with and without saliva. It was also established that EB@TiO_2 achieved effectiveness after 3 days of brushing. The composites also provide outstanding acid resistant stability. Despite this, and given the size of the sample used for the study, larger and longer duration of treatment would be required to conclusively determine the efficiency of EB@TiO_2 in reducing DH.

Acknowledgement

The financial support provided by the National Research Foundation of South Africa (No. 104824) is acknowledged by the authors.

Conflict of Interest: None.

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Manuscript II

Onwubu, S.C., Mdluli, P.S., Singh, S. and Obiora, C. C. 2019. The application of logistic equation model to predict the remineralization characteristics of desensitizing paste. International Journal of Dentistry **(Under-review)**.

The application of the logistic equation model to predict the remineralization characteristics of desensitizing paste

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Running Title: Mathematical modelling of desensitizing paste

Abstract

Objectives: A mathematical model making using of Verhulst logistic equation was developed to predict the remineralization behaviors of desensitizing paste.

Methods: The input parameter used for the model were obtained experimentally by brushing twenty-one simulated dentin specimens for seven days with three sample groups namely: EB@TiO_2, Colgate Pro-relief, and Sensodyne repair (n=7). Field Scanning Electron Microscope (FESEM) and ImageJ software were used to observe and measure the % occluded ratio of the dentin surface. The model fittings for the three sample groups were carried out using the built-in MATLAB Least-squares fitting routine fmincon in the optimization toolbox.

Results: The results suggest that the experimental parameter were in agreement with the model. It was found that the logistic equation model can make a future prediction of the remineralization pattern for EB@TiO_2 and Colgate Pro-relief. It was, however, found that the trajectory for the Sensodyne repair was a bit complex, thus making prediction difficult.

Conclusions: Overall, the salient feature of this study suggests that the logistic equation could be used to predict the remineralization behavior of desensitizing paste in the management of sensitive tooth.

Keywords: Desensitizing agents, Logistic Equation, Prediction, Remineralization

1. Introduction

Over the last decade, dentin hypersensitivity [DH] has been extensively researched owing to its widespread prevalence and noticeable painful oral health problem affecting many individuals (1). A previous study (2) reported that more than 80% of children and up to 43% of adult's population suffers from dental pain associated with DH. More worrisome is that DH negatively affects the quality of life of dental patients if left untreated (3). Consequently, numerous dentin remineralization strategies have been proposed in the literature (4, 5) for the management of DH. Amongst these, the use of biomaterials such as bioactive glass and proargin have been reported to effectively occlude the open dentinal tubules (6). While bioactive glass, for example, is noted to provide some substantial relief to patients (6), the overall duration of the treatment strategy using this material remain elusive in both saliva and without saliva.

Although bioactive glass occludes patent dentinal tubules by supplying calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions in an optimum oral environment to form Hydroxycarbonate Apatite (HCA) (7, 8), in some patients, however, particularly those with conditions of hyposalivation and xerostomia, the flow of saliva is limited (9). As reported in the literature (10, 11), saliva facilitates the deposition of the trap Ca^{2+} and PO_4^{3-} ions into open dentine tubules that gradually bring about tubule sealing or occlusion. Hence it sufficient to assume the effectiveness of the bioactive glass will be less effective in patients with a limited flow of saliva.

In an attempt to address the above concerns of limited salivary flow, proargin technology was developed by Kleinberg in 2002 based on the role saliva plays in naturally occluding dentinal tubules (6). According to (6), proargin comprises arginine (an amino acid with a pH 6.5-7.5), bicarbonate, pH buffer, and calcium carbonate. It is reported by Hamlin et al. (12) that the interaction of arginine and calcium carbonate at physiological pH subsequently attracts a calcium-rich layer that binds to the negatively charged dentin surface. This, in turn, facilitates the infiltration of calcium resulting in the blocking of the dentinal tubules (13). However, Yang et al. (14) found that Colgate Pro-relief showed no significant changes after treatment and immersion in artificial saliva for 14 days.

Given the differences in the occluding characteristics for the above biomaterials in saliva and without saliva, a new biomaterial from eggshell waste and titanium dioxide (EB@TiO_2) is proposed as an alternative occluding material for DH management. Whilst a recent study has demonstrated the occluding characteristics of EB@TiO_2 (15), the time required to completely and effectively occlude the dentine tubules in a simulated oral environment is yet to be established. Equally essential, and in line with the assertion of Schmidlin and Sahrmann (16), there is yet to be established a gold standard for DH management with a predictable and long-lasting relief of DH.

Importantly, mathematical modeling offers a different research perspective by overcoming some of the problems frequently encountered in an experimental study (17). Essentially, using numeral tools, Ilie et al. (17) assumed that it is possible to fashion out a controlled environment to address the challenges of long time period needed to effectively study the biological process. In the last decade, a different mathematical model has been proposed by various scholars to investigate dental hard tissues, most nevertheless, focus largely on dental caries and tooth demineralization process (17-19). Despite the numerous model developed to study dental tissues, there is limited evidence that suggests the use of a mathematical model to predict the remineralization potentials of desensitizing agents on dentin tubules. This study uses logistic equation model as a tool for the prediction of the effectiveness of desensitizing agents in occluding dentin tubules.

1.2 Logistic equation

The logistic equation was first proposed by the seminal work of Pierre-Francois Verhulst (1844-1845). Verhulst derived the logistic equation to describe the self-limiting growth of biological population (20). Interestingly, Sweilam et al. (21) assert that the logistic equation is described by a first-order ordinary differential equation. Their report resonates further with Murphy et al. (22) who noted that the logistic equation is formalized by the differential equation. Accordingly, it noted that the logistic model describes the growth of a population is limited by a carrying capacity of *b* (22). Hence, the logistic equation assumes that the growth rate decreases linearly with size until it equals zero at the carrying capacity (22).

Ever since the discovery, the logistic equation has been extensively used in many scientific fields such as ecology; chemistry; population dynamism; mathematical psychology; political science; geoscience; statistics; economics and sociology etc. (23-26). In ecology, for example, the logistic equation has been widely used to model the population growth where the rate of reproduction is proportional to both the existing population and the amount of resources available (21). This is expressed mathematically as follows:

$$\frac{dP}{dt} = rP\left(1 - \frac{P}{K}\right), \qquad (1)$$

where P represents population size, r is the constant that defines the growth rate, K is the carrying capacity, and t represents the time.

Another typical application of the logistic equation is in medicine, where the Logistic differential equation is used to model the growth of tumors or to study the reaction of pharmacokinetic (20). Here, the application of the logistic equation can be considered an extension of the above-mentioned use in the framework of ecology where d(t) the size of the tumor at time t (21). Given the predictive power of logistic equation, this study aimed to develop a mathematical model (logistic equation model) to study the remineralization capabilities of three desensitizing paste namely EB@TiO_2, proargin, and bioactive glass (Novamin) in saliva and without saliva.

2. MATERIALS AND METHODS

Food grade Anatese titanium dioxide (CAS No: 13463677) was purchase from Sigma-Aldrich (Germany). Citric acid monohydrate was supplied by Merck (South Africa). Two different brands of toothpaste namely: Sensodyne repair (GlaxoSmithKline, UK) and

Colgate Pro-relief (Colgate-Palmolive, Poland) were bought from a popular shopping mall located at Durban (South Africa).

2.1Preparation of Eggshell-Titanium dioxide composite (EB@TiO₂)

Eggshell and titanium dioxide composite was prepared in accordance with the method reported in literature (27). 20g of the fine eggshell powder were modified by adding 5g of anatase titanium dioxide (≤ 15μm). The mixture was subsequently ball-milled for 200 min to obtain eggshell-titanium dioxide composite (EB@TiO_2).

2.2 Experimental input parameter

The experimental parameter were obtained from the remineralization test conducted in our laboratory. Twenty-one dentin specimens measuring 5mm x 5mm x 1mm were prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water cooling conditions. Sensitive model were simulated by soaking the specimens in 4 % wt citric acid solution for 2 min. The specimens were then randomly assigned into three groups namely: EB@TiO_2, Colgate Pro-relief, Sensodyne repair (n= 7).

Each specimens were from the respective groups were brushed twice daily (morning and evening) for seven days with a toothbrush powered with 1.5v alkaline battery (Oralwise, China) for 1min and allowed to dry for 30s before rinsing with deionized water. Brushing were performed at room temperature using 100mg of each respective toothpastes. The slurry of EB@TiO_2 were prepared by mixing 100mg of the powder/200 μL of deionized water. After each brushing protocol, the specimens were immersed in saliva or without saliva. Using Field Scanning Electron Microscope (FESEM; Carl Zeiss) operating at controlled atmospheric conditions at 20 kV, we examined surface of the dentin after each day of brushing. The ratios of occluded tubules were computed using ImageJ software (National Institute of Health USA, http://imagej.nih.gov./ij). This was calculated by dividing the area of occluded tubules by the total tubules area using x 1500 magnification images (n=7). The mean values of the occluded area ratio were evaluated with 1-way analysis of variance (ANOVA). This was followed by a multi-comparison test with Bonferroni correction (α=.05)

2.3 Model Description

The mathematical model considers that the size of dentin tubules (S), the amount of calcium and phosphates deposits (A) influence significantly the time (t) needed to completely and effectively occlude the dentin tubules.

2.3.1. Logistic model

The following logistic equation model was proposed:

$$\frac{dX}{dt} = r_x X \left(1 - \frac{X}{K_x}\right), \tag{2}$$

where X is the percentage of tubules occluded, r_x the rate at which X is being occluded, K_x the maximum value of X and t is the time to complete remineralization of dentin tubules. Unless otherwise stated, we will take $K_x = 100\ \%$ since it is the maximum value of X.

2.3.2 Analytical solution to the model

By using the method of solving first order ordinary differential equation (separation of variable method) we obtain that the analytical solution of model equation 3 is:

$$X(t) = \frac{K_x\, C\, e^{r_x t}}{1 + C\, e^{r_x t}} \tag{3}$$

where $C = \frac{X(0)}{K_x - X(0)}$ and $X(0)$ is $X(t)$ at t = 0 (i.e., initial value of X). Further analyses on model (2) show that the model have two equilibrium points: the trivial equilibrium points $X^0 = 0$ and the positive equilibrium $X^* = K_x$. Conducting stability analysis about the two equilibrium states show that the positive equilibrium $X^* = K_x$ is globally stable. This is easily established as $X(t) \rightarrow X^* = K_x$ as $t \rightarrow \infty$. This results shows that it is possible for $X(t)$ to increase to the carrying capacity K_x. On the other hand, the trivial equilibrium point $X^0 = 0$ is unstable. This shows that it will be difficult for $X(t)$ to decrease to zero.

2.3.3 Model fitting and parameter estimation

A model fitting and parameter estimation was conducted using our model to fit real data for the three samples (EB@TiO_2, Colgate Pro-relief, Sensodyne repair) for the two cases: with saliva and without saliva. The aim of these analyses is to show that the model we considered can be used to study as well as make future predictions on these samples. For the model fitting, we take the carrying capacity (K_x) as 100% while the growth rate is estimated from the model fittings for all the samples. The model fittings were carried out using the built-in MATLAB Least-squares fitting routine fmincon in the optimization toolbox.

3 Results

3.1 Experimental parameter

Table 1 describes the % occluded area ratios of the dentin specimens brushed in seven days with or without artificial saliva. In the EB@TiO_2 treated group, the % occluded area ratios observed for the specimens with saliva were significantly higher than those without saliva for day 2, 3, 4, 6, and 7 (P<0.05). No differences observed between the two groups in day 5 (P>0.05). On the other hand, the group without saliva were higher than those with saliva on day 1 (P<0.05).

For the Sensodyne repair group, the % occluded area ratios observe in day 1-7 for the group with saliva were statistically significantly higher than those observed without saliva (P<0.05). In contrast, and for the Colgate Pro-relief group, the specimens brushed without saliva were consistently and significantly higher when compared against the group with saliva in each respective days (P<0.05).

Table 1: Mean, standard deviation for the area ratios of the occluded tubules brushed in seven days with and without saliva

	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	WS	S	WS	S	WS	S	WS	S	WS	S	WS	S	WS	S
EB@TiO2	19±2.6^{a}	15.6±2.2^{b}	35.1±4.4^{a}	43.6±4.7^{b}	50.4±4.3^{a}	71.4±2.8^{b}	62.0±3.7^{a}	82.0±3.1^{b}	93.3±2.3^{a}	93.9±3.0^{a}	95.1±1.6^{a}	98.4±1.9^{b}	97.9±1.3^{a}	99.3±1.0^{b}
Colgate Pro- relief	32.3±3.0^{a}	16±3.6^{b}	45.7±6.8^{a}	21.4±2.2^{b}	62.3±6.7^{a}	20.3±2.7^{b}	62.0±6.7^{a}	20.9±2.9^{b}	68.6±6.1^{a}	19.9±3.4^{b}	74.3±6.8^{a}	61.9±4.8^{b}	88.9±3.2^{a}	80.1±8.6^{b}
Sensodyne repair	12.9±3.6^{a}	19.3±3.5^{b}	13.6±2.5^{a}	29.0±2.2^{b}	13.4±2.8^{a}	44.1±4.9^{b}	15.0±3.3^{a}	60.9±4.6^{b}	16.0±3.1^{a}	55.3±5.3^{b}	17.0±3.7^{a}	53.3±6.2^{b}	70.1±4.2^{a}	90.4±3.0^{b}

Values are mean ± standard deviations (n=7). Different superscript letters indicate significant differences (P<0.05). WS=without saliva, S=with saliva

3.2 Predictability of the model

The results of the model fitting for the three samples are presented in Figure 1. From the Figure 1, it can be observed that the proposed model produce a good fitting for two samples: EB@TiO_2 and Colgate Pro-relief (for the two cases: with and without saliva), but did not produce a very good fitting for the Sensodyne repair.

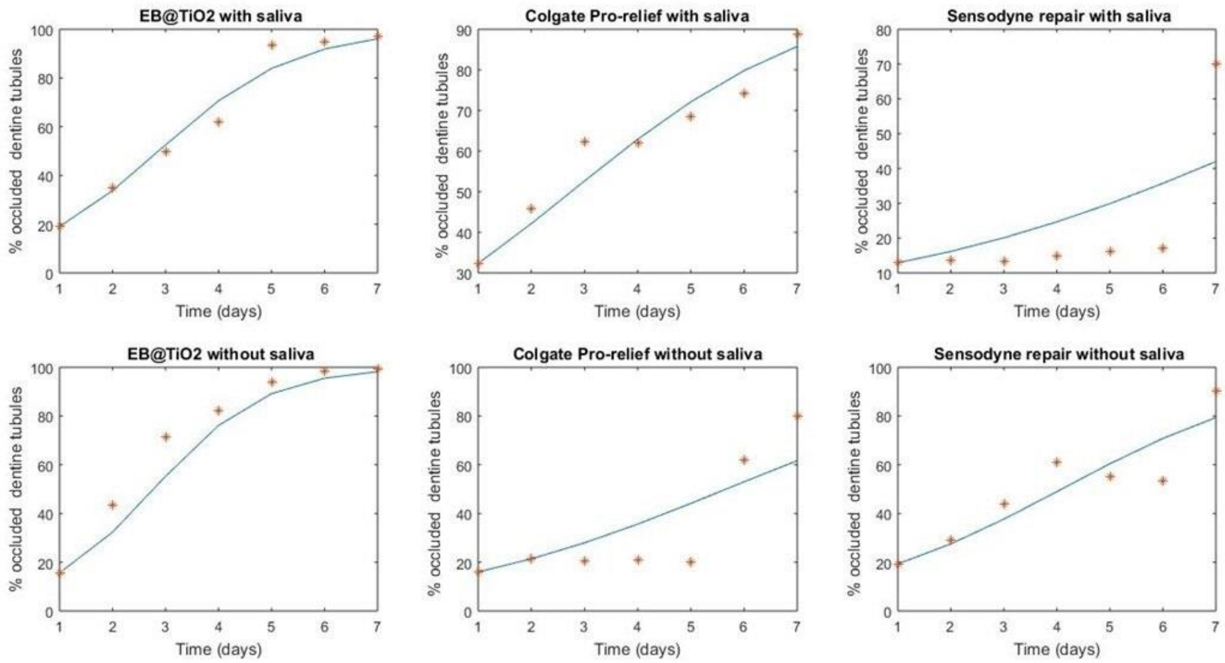

Figure 1: Model fitting of the desensitizing paste in saliva and without saliva

The estimated parameters for tubule occlusion rate (r_x) is given in Table 2. For the specimens treated with EB@TiO$_2$, it was observed that the r_x were higher (0.9500) with saliva when compared with the rate measured without saliva (0.7762). Similarly, the r_x measured for Sensodyne repair was higher in the specimens treated with saliva (0.4635) when compared against those treated without saliva (0.2646). In contrast, it was found that the r_x for specimens treated with Colgate Pro-relief without saliva was higher than those measured with the specimen treated with saliva.

Table 2: Estimation of model parameters

Parameters (r_x)	Estimated by model fitting	
	Without saliva	With saliva
EB@TiO$_2$	0.7762	0.9500
Colgate Pro-relief (Proargin Technology)	0.4230	0.3558
Sensodyne repair (NovaMin)	0.2646	0.4635

4. Discussion

The purpose of this study was to investigate the use of the logistic equation model to predict the remineralization characteristics of desensitizing paste. Ilie (19) had advised that the parameters needed for developing a mathematical predictive model should be determined experimentally. In line with the author's advice, the input parameters used for the logistic equation model were determined through the remineralization test conducted on prepared bovine specimens. The findings suggest that the experimental results were in agreement with our model prediction.

Overall, the remineralization characteristics measured for EB@TiO_2 were significantly better than those observed for Colgate Pro-relief and Sensodyne repair ($P<0.05$). It was found that the remineralization behavior of EB@TiO_2 in saliva and without saliva appear to follow the same pattern. More importantly, the model produces a good fitting for the dentin specimens treated with or without saliva (Figure 1). Predictably, and regardless of the salivary condition, it is assumed that EB@TiO_2 would achieve complete occlusion of the dentin tubules at the end of seven days brushing. The remineralization ability observed for samples treated with EB@TiO_2 may be attributed to the modification of the carbonate structure content in eggshell with titanium dioxide. This is in agreement with the suggestion of Cutler (28) that nanosized titanium dioxide together with abrasive materials promotes the occluding of dentin tubules. Another reason for the effective remineralization seen in the EB@TiO_2 sample group could be attributed to the nanosized particle distribution in EB@TiO_2 (29). According to Nakashima et al. (30), nanosized calcium carbonate materials have unique high surface energy–thus facilitating the attachment of calcium-rich ions on the oral tooth surface.

With regards to the Colgate Pro-argin paste, the model fitting suggests that the behavior with and without saliva differs. It was found that the model produced a better fitting for the samples treated without saliva (Figure 1). In addition, it is predictable that Colgate Proargine would achieve a fast and complete occlusion of the dentin tubules after seven days of treatment. However, the model pattern for the samples treated with saliva immersion suggests that the effectiveness of pro-argin would require a longer period than seven days to effectively occlude the tubules. This is in agreement with the recent clinical finding that pro-argin achieved significant relief of DH after application for 24weeks (3).

In terms of the Sensodyne repair, we could not achieve a good model fit to make an accurate prediction of the treatment. This may, however, be related to the quality of the input parameters (19). Equally important, it was worth mentioning that the calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions in NovaMin are protected by glass particles and thus would need to be trapped for it to effectively penetrate the dentin tubules (31). The absence of saliva in the treated samples may have contributed to the complex trajectory behavior observed. In saliva, it is assumed that the trapped calcium and phosphate ions may be delayed in the release which could also have accounted for the complex trajectory observed for the samples treated with saliva immersion.

5. Conclusion

In this paper, we have demonstrated that our model can be used to study and make a future prediction for the two samples: EB@TiO_2 and Colgate Pro-relief (for both cases: with and without saliva). For the Sensodyne repair, we discover from the figure that the trajectory for its real data is a bit complex. This makes it difficult to obtain a good fitting using our model. We hope to consider a more complex model that can fit the real data for Sensodyne repair in our future work.

Acknowledgment

The authors acknowledged the financial support from the National Research Foundation of South Africa (No. 104824).

Conflict of Interest: The authors declare that they have no conflict of interest.

Data Availability Statement

The data used to support the findings of this study are included within the article.

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Manuscript III

Onwubu, S.C., Mdluli, P.S., Singh, S. and Nyembe, S. 2019. Evaluating the abrasivity of a nanosized eggshell-titanium dioxide on tooth enamel. Clinical Oral Investigations **(Under-review**).

Evaluating the abrasivity of a nanosized Eggshell-titanium dioxide on tooth enamel

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Abstract

Objectives: The purpose of this study was to evaluate the abrasivity of a nanosized eggshell-titanium dioxide (EB@TiO_2) in comparison with calcium carbonate and hydrated silica containing toothpaste.

Material and Methods: Thirty-five prepared bovine tooth enamel specimens were randomly assigned to 5 sample groups (n=7): 1 Colgate regular; 2 Colgate Pro-relief; 3 Sensodyne rapid relief; 4 Sensodyne repair; 5 EB@TiO_2. Half of the enamel surface was brushed with each respective sample groups while the other half was covered with a tap. The mean roughness value (R_a) of the brushed and covered halves were measured with an Atomic Force Microscope (AFM). Scanning Electron Microscope (SEM) was used to evaluate the surface morphology and changes. Pair sample test and ANOVA was used to analyze the mean roughness value (R_a) for all specimens. A Bonferroni correction was used to identify the mean differences among the 5 groups (α=.05).

Results: The finding from this study reveal that all the tested toothpaste significantly abraded the enamel ($P<0.05$). The abrasivity found in calcium carbonate containing toothpaste were lower than the hydrated silica toothpaste. Overall, Colgate regular had the lowest toothpaste abrasivity, followed by EB@TiO_2 while Sensodyne rapid relief had the most enamel wear.

Conclusion: The salient feature of this study suggest that EB@TiO_2 is suitable for oral use, as its abrasivity is comparable with calcium carbonate containing toothpaste.

Clinical relevances: An abrasive material made from eggshells would be important in the prevention of tooth abrasion from toothpaste abrasivity that is considered a dental problem.

Keywords: Abrasivity, enamel, toothpaste, Atomic force microscope

1. Introduction

Over the last decade, there has been a significant improvement in the oral health of the general population worldwide. Much of this improvement could be attributed to the daily use of toothpaste as an effective home oral care products [1]. Traditionally, toothpaste is a paste or gel that is often used in combination with a toothbrush to maintain and improve oral health and aesthetics [2]. Since the introduction of toothpaste by the Egyptian around 3000-5000 BC, the formation of toothpaste has evolved from the use of powdered ox hooves ashes, myrrh, eggshells, and pumice with the aim to remove debris from teeth - to a more complex ingredient [2]. In the last decade, modern toothpaste formulation has been manipulated to deliver chemical and physical mediated benefits such as the prevention of caries and supra-gingival plaque, calculus removal, extrinsic stain removal, and the treatment of dentine hypersensitivity [3, 4].

Importantly, modern toothpaste contain abrasives that aid tooth cleaning and whitening, flavors (for fresh breath) and colorant to enhance their visual appeal [2]. According to the aforementioned author, abrasives are the most traditional toothpaste excipient and contribute secondarily to toothpaste rheology [2]. Ideally, abrasive particles are harder than the stain but softer than sound enamel, a stain can be removed without causing significant damage to the tooth surface [5]. This is highly important as excessive abrasive material could abrade tooth surface away, resulting in undesirable tooth wear [6]. According to Lippert [2], the abrasive cleaning process is affected by various key parameters, such as particle hardness; shape; size; size distribution; concentration of its particles; and applied load during brushing. Accordingly, the main objectives of toothpaste manufactures are to optimize this formulation for cosmetic and oral health benefits [7]. In light of these, toothpaste manufacturers often utilized calcium carbonate, dicalcium phosphate, silica, alumina oxide, calcium pyrophosphate, sodium metaphosphate, perlite, nano-hydroxyapatite, and sodium bicarbonate etc. to provide an abrasive action [2, 6]. Concerning, and as highlighted by Field [8], the action of said materials are, however, complex and chemically identical components may result in different abrasion characteristics.

Particularly, early studies [9, 10] have shown that the abrasivity of toothpaste could significantly influence the rate of abrasion than the features of the brush alone. Added to these, it has been reported that modern toothpaste due to their complex formulation has different abrasivity values [8]. Hence an understanding of their clinical applications, effectiveness and safety concern is highly paramount to prevent surface loss in both enamel and dentine [6, 11]. According to Anusavice and Antonson [12], the abrasiveness of toothpaste may adversely impact on the exposed cementum and dentine surfaces. Equally worrisome, Bizhang, Riemer, Arnold, Domin and Zimmer [13] reported that toothpaste with high relative dentine abrasivity (RDA) values resulted in greater losses of dentine. Corroborating further, Arnold, Gröger, Bizhang and Naumova [14] reported that the abrasivity of toothpaste have an adverse effect on the occlusion of dentine tubules which could reopen the tubules during brushing procedure.

Moreover, Addy [15] caution that the abrasivity of toothpaste products has the potential to cause tooth wear, which could manifest dentine hypersensitivity arising from the exposed dentine. This is concerning as there is a strong correlation between the abrasiveness of the toothpaste, tooth brushing, and the occurrence of acute trauma, caries and periodontal disease, and dentine abrasion [8, 15]. Anusavice and Antonson [12], therefore, emphasized that toothpaste does not have to be highly abrasive to clean teeth effectively. Hence, the ideal toothpaste should provide the greatest possible cleaning action on tooth surfaces with the lowest possible abrasion rate [6, 12]. Essentially, the ‘holy grail’ for manufacturers of abrasives and toothpaste manufacturers alike is to make toothpastes that clean well and at the same time; virtually non-abrasive to the dental hard tissues [2].

Recently, the use of eggshell powder has attracted much interest due to its relatively low abrasivity. Onwubu, Vahed, Singh and Kanny [16] demonstrated that a dental abrasive material made from eggshells was effective in the polishing of dental acrylics. Chen [17], on the other hand, revealed that eggshells of particle size between 0.1 μm to 10 nm produced an ultra-fine powder, which made them useful in toothpaste and cosmetics. In this study, we aim to evaluate the abrasivity of new toothpaste formulation containing modified eggshell powder and titanium dioxide (EB@TiO2) in comparison with some commercial toothpaste. The null hypothesis stated was that there is no significant difference in the abrasivity of EB@TiO_2 and the different toothpaste.

2. MATERIALS AND METHODS

Anatase titanium dioxide (CAS No: 13463677) was purchased from Sigma-Aldrich (Germany). Four different brands of toothpaste namely: Sensodyne repair, and rapid relief (GlaxoSmithKline, UK), and Colgate regular and Colgate Pro-relief (Colgate-Palmolive, Poland) were bought from a popular shopping mall located at Durban (South Africa).

2.1 Preparation of Eggshell-Titanium dioxide (EB@TiO_2)

Eggshell and titanium dioxide composite were modified in accordance with the method reported by Onwubu, Mdluli, Singh, Lawrence and Ngombane [18] following two steps. In the first step, 30g of the dried eggshell were placed in a 500ml stainless jar (inner diameter of 100 mm), together with 50 stainless steel balls of 10mm diameter and dry-milled in a planetary ball mill (Retsch PM 100) at 400 rpm for 20 min. The collected powder was sieved to a particle size of ≤ 25μm using a mechanical sieving shaker (Retsch AS 200, Germany). Thereafter, 20g of the fine eggshell powder obtained in step 1 was modified by adding 5g of anatase titanium dioxide (≤ 15μm). The mixture was subsequently ball-milled for 200 min to obtain eggshell-titanium dioxide composite (EB@TiO_2). It is worth mentioning that an extensive detail of the particle size, shape, phase, and cytotoxicity of EB@TiO_2 are reported in another published papers [18-20].

2.2 Assessing the abrasivity of EB@TiO_2

The abrasiveness of EB@TiO_2 on tooth enamel was measured in vitro using a simulated brushing protocol (Figure 1). Thirty-five bovine enamel measuring 5mm x 5mm x 3mm was prepared by sectioning perpendicular to the long axis of the teeth below the enamel-dentinal junction using a low-speed diamond saw under water cooling conditions. Thereafter, enamel disc was embedded in a resin (AMT composite, South Africa). Silicone mold (Silicone rubber mold; Agar scientific) was used to make a mounting base.

Before brushing, the samples were kept in artificial saliva for 5min to simulate oral condition. One half of the tooth was then covered with aluminum tape. Tooth brushing was perform using a powered 1.5v alkaline battery (Oralwise, China) for 2min before rinsing with deionized water. Brushing was performed at room temperature using

100mg of each respective toothpaste (Table 1). The tooth brushing protocol load was 200g. After the brushing protocol, the tape was subsequently removed.

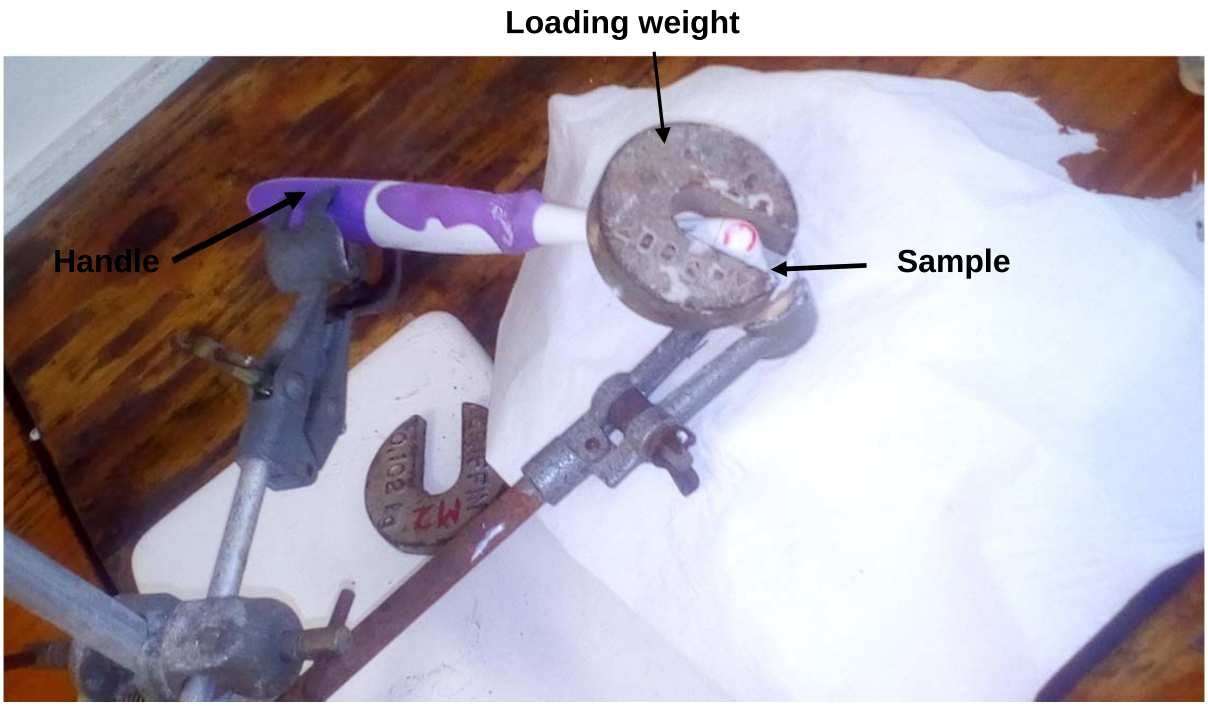

Figure 1: Brushing protocol apparatus set-up.

Table 1: Toothpaste and manufacturers

Sample	Abrasive ingredients	RDA value*	Manufacturer
Colgate (Regular)	Calcium carbonate, sodium bicarbonate	68 [21]	Colgate-Palmolive
Sensodyne Rapid relief	Hydrated silica, Pentasodium Triphosphate	70 [14]	GlaxoSmithKline
Colgate Pro-Relief	Pro- Arginine, Calcium carbonate, sodium bicarbonate	30	Colgate-Palmolive
Sensodyne Repair	Hydrated silica, Calcium Sodium Phosphosilicate (NovaMIN)	119 [14]	GlaxoSmithKline
EB@TiO_2	Eggshells and Titanium dioxide	N/A	Researcher (s)

2.3 Atomic Force Microscope (AFM) analysis

The height differences between the covered halves and the brushed halves of the enamel disc were measured using AFM (Nanoscope; Bruker). The instrument was set in a contact mode with a scanning size of 5 µm, and a scan rate of 2.441 Hz. For each specimen, 5 different measurements of the mean surface roughness R_a values were made. The average measured R_a values obtained were then used for the statistical analysis.

2.4 Statistical Analysis

The mean differences between the brushed and the covered halves of the various sample groups were compared using pairwise tests. In addition, the mean values of the height differences were further compared between the different toothpaste with 1-way analysis of variance (ANOVA) by means of statistical software (IBM SPSS Statistics v25; IBM Corp.), followed by Bonferroni correction and the significance level was set at $\alpha = 0.05$.

2.5 Field Scanning Electron Microscope observation

The surface of the brushed and covered halves of the specimens was characterized using Scanning Electron Microscope (FESEM; Carl Zeiss). As a proxy measure, a specimen from each group was analyzed microscopically. The FESEM was operated at controlled atmospheric conditions at 5kV.

3. Results

3.1 Atomic Force Microscopy (AFM)

The pairwise comparison test of the brushed and covered halves of the tooth is shown in Table 2. A statistical difference was observed between the brushed and covered halves of the tooth specimens from each of the respective toothpaste ($P<0.05$). Despite this, the specimens brushed with Colgate toothpaste had the least abrasive R_a mean wear gap (Ra = 8 nm), followed by EB@TiO_2 (R_a =16 nm). The highest mean wear gap (R_a= 23 nm) were found in the specimens brushed with Sensodyne rapid relief.

AFM micrograph shown in Figure 2 further depicts the surface profile of the brushed and covered halves of the specimens. The surface profile evidently reveals the differences between the two halves with the different toothpaste. Notably, the covered halves (A-E) of the specimens appeared rougher than the brushed halves (A1-E1).

Table 2: Paired sample test between the brushed and covered halves

Sample groups	Abrasivity R_a value		Wear Gap	Sig.
	Covered halves	Brushed halves		
Colgate	27.0±6.2	19.5±2.6 nm	8 nm	0.008
Colgate Pro-relief	23.8±6.6	6.4±1.0 nm	17.4 nm	0.001
Sensodyne repair	29.6±12.3	7.2±1.6 nm	22.4 nm	0.003
Sensodyne rapid relief	33.5±6.6	10.5±2.2 nm	23 nm	0.000
EB@TiO2	25.6±5.9	9.6±3.4 nm	16 nm	0.002

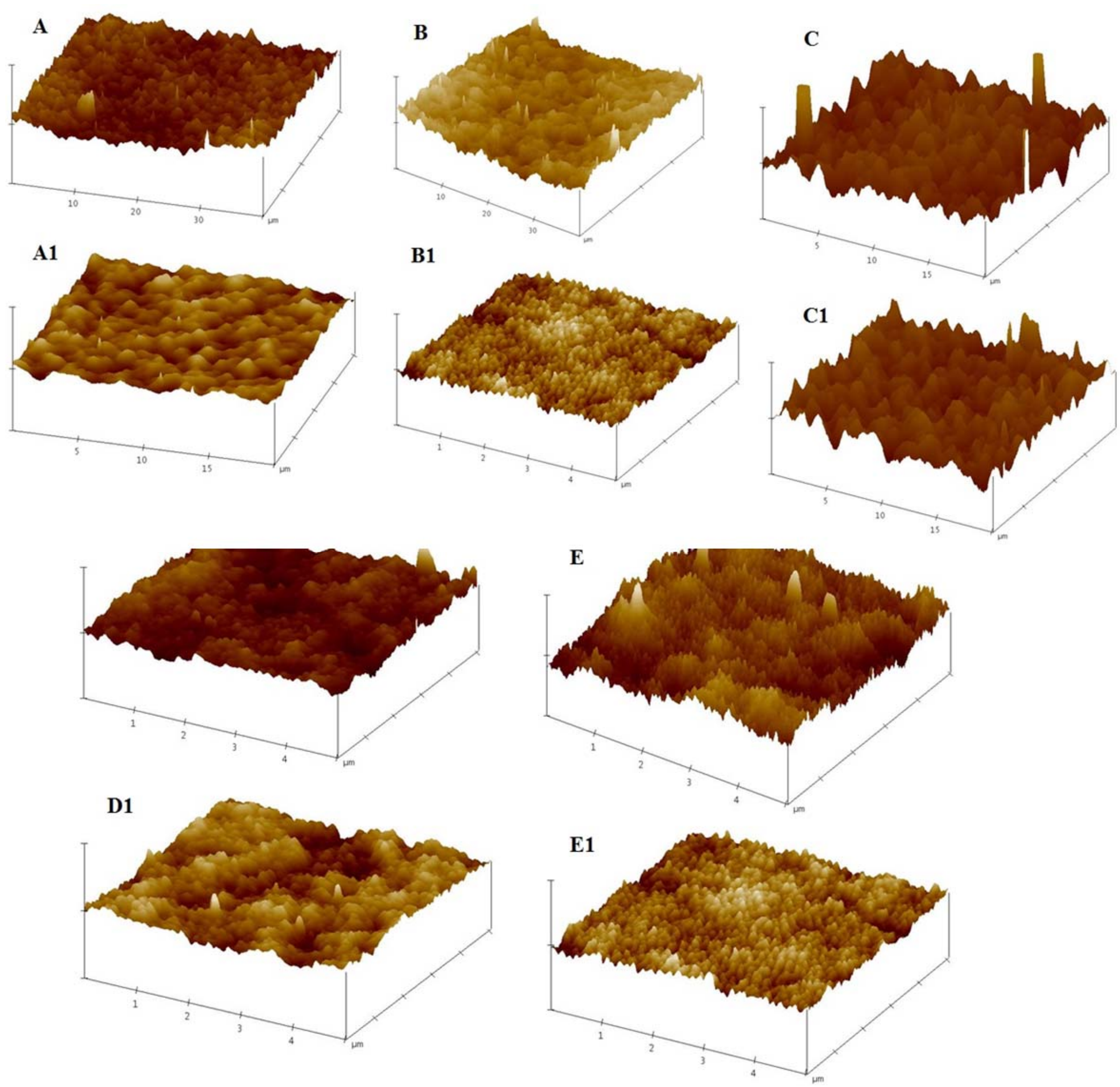

Figure 3. AFM profile of bovine specimen brushed with (A1) Colgate; (B1) EB@TiO_2; (C) Colgate Pro-relief; (D) Sensodyne Repair (E) Sensodyne rapid relief.

The 1-way ANOVA, mean, standard deviation, and standard error results are given in Table 3. A statistically significant difference was measured in the mean surface roughness R_a values of bovine tooth specimens and their interactions with the different toothpaste used for the brushing protocol ($P<0.05$). Moreover, the mean R_a values of the bovine specimens brushed with Colgate (Regular) were significantly lower than those of the specimens brushed with Sensodyne Repair, and Sensodyne rapid relief ($P<0.05$). No statistical significant differences were measured between the mean R_a values of Colgate (Regular) and EB@TiO_2, and Colgate Pro-Relief ($P>0.05$).

Table 3: Abrasivity of the tested toothpaste

Sample groups	N	Mean	SD	SE	ANOVA	Bonferroni Post Hoc test
Colgate (Regular)	7	7.5	5.2	2.0	0.010	1.000
Colgate Pro-relief	7	17.3	7.2	2.7		0.329
Sensodyne Repair	7	22.3	12.1	4.6		0.020
Sensodyne rapid relief	7	23.0	6.9	2.6		0.014
EB@TiO2	7	16.1	8.0	3.0		0.611

3.2 Field-Scanning Electron Microscope observation of specimens

The FESEM images of the specimens are given in Figure 3. The images visibly confirmed the surface roughness differences between the brushed (A1-E1) and covered halves (A-E) of the specimens and their interaction with differently tested toothpaste. A noticeable difference in the surface roughness of the covered and the brushed halves is evident for all the tested toothpaste.

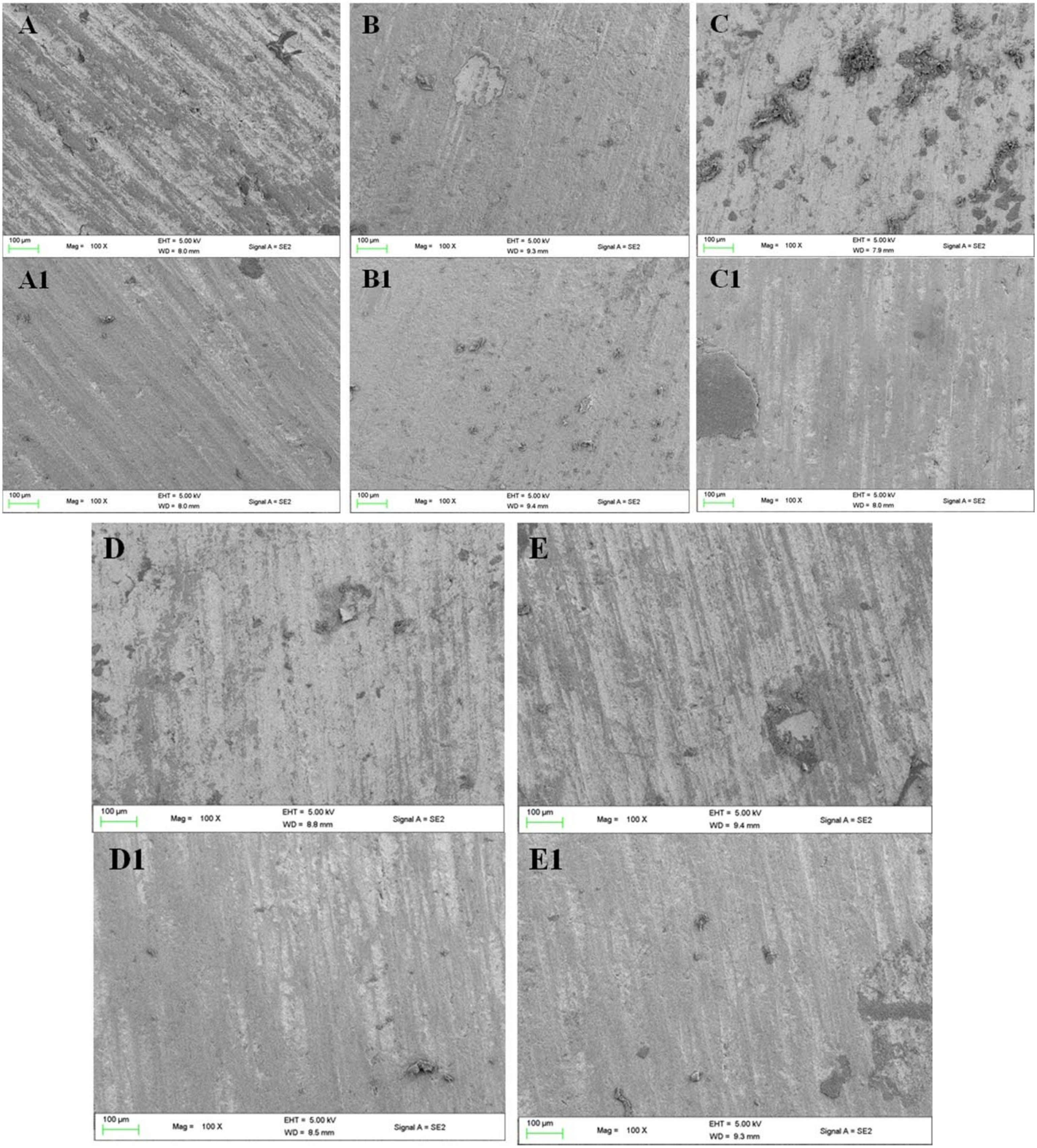
A
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 8.0 mm
Signal A = SE2
B
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 9.3 mm
Signal A = SE2
C
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 7.9 mm
Signal A = SE2
A1
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 8.0 mm
Signal A = SE2
B1
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 9.4 mm
Signal A = SE2
C1
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 8.0 mm
Signal A = SE2
D
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 8.8 mm
Signal A = SE2
E
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 9.4 mm
Signal A = SE2
D1
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 8.5 mm
Signal A = SE2
E1
100 µm
Mag = 100 X
EHT = 5.00 kV
WD = 9.3 mm
Signal A = SE2

Figure 3: FESEM images of bovine surface brushed with (A1) Colgate; (B1) EB@TiO_2; (C) Colgate Pro-relief; (D) Sensodyne repair (E) Sensodyne rapid relief (x100 magnification; A-E represents covered halves of the tooth).

4. Discussion

Dental abrasion from toothpaste abrasives is reported to be a leading cause of a dental problem in the general population [21]. As a consequence of this, the measurement and standardization of toothpaste abrasive are highly pertinent for the prevention of tooth abrasion [21]. The purpose of this study was to evaluate the abrasivity of a modified nanosized eggshell-titanium dioxide (EB@TiO_2) dental abrasives. To fully quantify the abrasivity of toothpaste, different techniques have been reported in the literature [14, 22, 23]. In this study, atomic force microscope (AFM) was used to quantitatively and qualitatively measure the abrasivity of EB@TiO_2. The mean roughness values (R_a) obtained were used for statistical analysis. Based on the study results, the tested hypothesis was accepted. The findings reveal that the different toothpaste used significantly abraded the tooth enamel specimens (P<0.05).

Moreover, it was found that the R_a values measured for EB@TiO_2 were not significantly different from other tested toothpaste (P>0.05). It can, therefore, be inferred that EB@TiO_2 is suitable as a dental abrasive for use in toothpaste formulations. Although the early study by Harris [24] had suggested that TiO_2 has a high Mohs hardness and thus abrasive, the results from this study, however, found that R_a measured for EB@TiO_2 were comparable with other calcium carbonate containing toothpaste (Colgate regular and Colgate Pro-relief). This, therefore, supports the findings by Onwubu, Mdluli, Singh, Lawrence and Ngombane [18] that the modification of eggshell with titanium dioxide does not negatively impact on the calcium carbonate structure.

Nonetheless, the R_a values measured for the calcium carbonate based toothpaste (Colgate regular, Colgate Pro-relief, and EB@TiO_2) were lower when compared against the hydrated silica containing toothpaste (Sensodyne repair, and Sensodyne rapid relief). The difference measured between toothpaste containing calcium carbonate as the abrasive material and hydrated silica may be attributed to a difference in the RDA values (Table 1). According to Hunter, Addy, Pickles and Joiner [25], although calcium carbonate and hydrated silica are chemically identical, nevertheless, they can produce different cleaning/ abrasion characteristics. Equally important, an in agreement with this study, several other studies [26, 27] reported calcium carbonate toothpaste showed lower abrasivity when compared against hydrated silica containing toothpaste.

Furthermore, the findings from this study revealed that the abrasivity of Colgate regular was significantly lower when compared against Sensodyne rapid relief, and Sensodyne repair, respectively (P<0.05). Specifically, the lowest abraded toothpaste measured were found in the specimens brushed with Colgate regular (7.5±2.0), followed by EB@TiO_2 (16.1±3.0) while Sensodyne rapid relief had the highest (23.0±2.6) abraded enamel surface (Table 3). This finding agrees with Schemehorn, Moore and Putt [28] that toothpaste containing hydrated silica have higher abrasivity on tooth enamel. On the contrary, Ferreira, Ramos-Jorge, Delbem and Vieira [29] found no significant difference between calcium carbonate and silica containing toothpaste. The difference between these studies may be attributed to the treatment condition, and or tested toothpaste. For example, Ferreira, Ramos-Jorge, Delbem and Vieira [29] acknowledged that the similarities observed in the abrasivity of calcium carbonate and silica were due to the softened surface which is easily abraded irrespective of the abrasive found in the toothpaste material.

Added to the above, Hunter, Addy, Pickles and Joiner [25] noted that a mixture of chemically different abrasives could result in effects differing from those of the individual component. Arguably, the presence of other active chemicals such as pentasodium triphosphate in Sensodyne rapid relief, and calcium sodium phosphosilicate in Sensodyne repair could have contributed to their increase abrasiveness (Table 1). Overall, the AFM images (Figure 2) and the SEM images (Figure 3) provide visible evidence that the brushed and the covered halves (unbrushed) were significantly different. This further supports the work of Athawale, Srinath and Chowdary [30] who observed a significant difference in the enamel abrasivity before and after brushing, albeit, with a different toothpaste. Regardless of these findings, in situ studies have shown that the amount of enamel loss from toothpaste abrasion is clinically negligible [31], and thus all the different tested toothpaste could be considered safe.

5. Conclusion

The prominent aspect of this study reveals that all the tested toothpaste significantly abraded the enamel after brushing. The abrasivity of EB@TiO_2 was however comparable to other tested calcium carbonate containing toothpaste. This study conclusively suggests EB@TiO_2 is suitable for use in toothpaste formulation.

Compliance with Ethical Standards

Conflict of Interest: Onwubu SC declares that he has no conflict of interest. Mdluli PS declares that he has no conflict of interest. Singh S declares that he has no conflict of interest. Mokgobole MU declares that he has no conflict of interest. Bharuth V declares that he has no conflict of interest.

Funding: The work was supported by the National Research Foundation of South Africa (No. 104824)

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors./All applicable international, national, and/or institutional guidelines for the care and use of animals were followed./All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: For this type of study, formal consent is not required. /Informed consent was obtained from all individual participants included in the study.

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Chapter Five – Critical review

In the last decade, there has been an unprecedented advancement in oral health care. Much of these advancements was attributed to the development of oral hygiene products, which have contributed to the improvement and maintenance of oral health (Abhay, Dinnimath and Hullatti 2014; Cummins and Marsh 2018). Baglar *et al.* (2018) reasoned that public awareness and coupled with the wide usage of caries prevention products have ultimately increased the remaining of teeth in the mouth without caries. Further to this, due to improved healthcare globally, more people are retaining more vital and minimally restored teeth (Douglas-de-Oliveira *et al.* 2018). This has, however, resulted to an increase in the number of non-carious dental problem such as tooth erosion, attrition, abfraction, abrasion, and or gingival recession, thus contributing to the high prevalence of dentin hypersensitivity (Arnold, Prange and Naumova 2015). More worrisome is that dentin hypersensitivity [DH] has the capacity to negatively impact on an individual's quality of life such as their social factors, psychological status, and or discomfort from pain (Bekes and Hirsch 2013).

Given the above concerns, several products or agents are available in the market with a claim to effectively reduce the incidence of DH (Davari, Ataei and Assarzadeh 2013; Teke, Enongene and Tiagha 2017). Nevertheless, it is reported literature that there is yet to be a gold standard material to effectively manage the condition (Schmidlin and Sahrmann 2013; de Melo Alencar *et al.* 2019). Bearing this in mind, this study aim was to develop and evaluate the quality of a modified nano-sized eggshell and titanium dioxide (EB@TiO2) composite as the active ingredients in the management of DH. The hypotheses tested in this study were:

Hypothesis one:

> H_0: There will be no difference in the dentine tubules occlusion pre- and post-acidic exposure of the specimens treated with EB@TiO_2.
>
> H_1: There will be significant difference in the dentin tubules occlusion pre- and post-acidic exposure of the specimens treated with EB@TiO_2 desensitising toothpaste.

Hypothesis two:

H_0: There is no significant difference in the protective effect of EB@TiO_2 against erosive acids when compared against commercially available toothpastes.

H_1: EB@TiO_2 will offer more protection against erosive acids compared to commercially available toothpastes.

Hypothesis three:

H_0: There are is no significant difference in the abrasion rate of EB@TiO_2 and commercially available toothpastes on tooth enamel.

H_1: There are significant differences in the abrasion rate of EB@TiO_2 and commercially available toothpastes on tooth enamel.

5.1 Research hypothesis one: Evaluating the acid resistance properties of EB@TiO_2

Enamel erosion has been identified as one of the main predisposing factors that contribute to the onset of DH (West, Seong and Davies 2015). Although some over the counter (OTC) commercially available toothpaste has shown promising results in the management of DH (Hirsiger *et al.* 2019), their effectiveness in the acidic environment is compromised, resulting in DH relapse (Arnold, Prange and Naumova 2015; Yu *et al.* 2016). In light of the aforesaid concern, it becomes pertinent to evaluate the protective effect of EB@TiO_2. Paper I, II, III, and IV were based on the protective effect of EB@TiO_2 and some commercially OTC toothpaste against erosive acids. While bovine tooth has been suggested as an alternative for the human tooth in both in vitro and in situ experiments, the acid resistance of OTC toothpaste was evaluated using an eggshell model (Paper I, II, and III). The motive for this new approach was premised on the chemical composition of eggshells that has similar characteristics to the natural bones and teeth (Paper I).

Naturally, an acidic attack on tooth enamel results in the dissolution of the hydroxyapatite (HA) crystals (Featherstone and Lussi 2006). According to the chemical process model, the hydrogen ions (H^+) derived from strong/weak acids binds or complex calcium which facilitates the removal of calcium ions from the HA crystal surface (Featherstone and Lussi 2006). Given this scenario, it was assumed that the acid attack on eggshell would cause the dissolution of the carbonate into calcium oxide and carbon dioxide (gas). Hence Paper I, II, and III reported on a new technique to determine the acid resistant properties of toothpaste. The eggshell model was developed by doping both eggshells (Paper I), and EB@TiO_2 (Paper II and III) into the respective toothpaste. A gas pressure sensor was used to measure the amount of carbonate dissolution by measuring the pressure (kPa/s) in the carbon dioxide released. While this technique can be considered novel to study enamel erosion, its use in other areas of science have been widely reported in the literature (Stary 2004; Sridar *et al.* 2016; Foust 2018; Krehbiel *et al.* 2019). As highlighted in the Vernier LabPro Manual (2008: 19), the pressure sensor can accurately measure carbon dioxide. Added to this, a gas displacement technique was also used to support the pressure sensor data (Paper I).

Paper I established that eggshell model could be used to study the acid resistant properties of toothpaste. The EDX analysis of the model pre and post doping revealed a significant difference in the elemental composition (Table 2; Paper I). More so, the SEM morphology of the eggshell model confirmed a hydroxyapatite-like structure. It was found that all the tested toothpaste (Figure 8; Paper I) were effective against erosive attacks. However, a salient point worth emphasising is that the eggshell alone without the protective covering of toothpaste had the highest dissolution of the carbonate structure (Figure 8 and 9; Paper I). This aligned to the solubility of hydroxyapatite structure of the enamel and dentin in acidic substance (Featherstone and Lussi 2006), thus supporting the preventive benefits of oral care products such as toothpaste in the maintenance of oral health (Kato *et al.* 2010).

While eggshell is reported to be the future of teeth remineralisation, from the study (Paper I), it is sufficient to assume that eggshell alone would not be effective in the long term management of DH. Hence, it was pertinent to modified eggshell with titanium dioxide. The motive for the use of titanium was the high acidic resistance and antibacterial properties (Montazer *et al.* 2011; García-Valverde *et al.* 2014). Equally important, a calcium carbonate modified titanium with improved acidic resistant properties have been successfully used in the papermaking industry (Tao, He and Zhao 2015). Paper II, III and IV evaluated the acid resistance of eggshell-titanium dioxide composite. The composite was prepared using a mechanochemical approach. This technique is reported to be gaining increasing attention among researchers due to their simplicity, low cost, reproducibility and environmental friendliness (Baláž 2018). The milling condition of 20g of eggshell powder and 5g of titanium dioxide milled at a speed of 450 rpm for 200min were based on the parameter reported in the literature (Paper II).

The success of the modification was confirmed using different traditional characterisation technique such as Field Scanning Electron Microscope (FESEM), X-ray Diffraction (XRD), Fourier Transform Infrared (FTIR), and High Transmission Electron Microscope (HRTEM). Paper II established that acid resistance properties of EB@TiO_2 were significantly better than eggshell alone. It was reported that the amount of carbon dioxide release in the toothpaste doped with EB@TiO_2 were consistently lower than those doped with eggshell alone (Table I; Paper II). The study confirmed that modifying eggshell with titanium dioxide improved its acidic resistance characteristics.

Furthermore, the buffering characteristics of EB@TiO_2 at different pH were compared with eggshell alone and OTC toothpaste (Paper II). According to Moron *et al.* (2013), the pH of the oral environment can affect the protective effect of fluoride-containing OTC toothpaste. As a consequence, fortifying with calcium or calcium-containing product was suggested in the literature (Joiner *et al.* 2009). It was demonstrated in Paper II that the buffering characteristics of EB@TiO_2 were comparable with eggshell alone at pH 2, 4, and 5, whilst more superior to the tested OTC toothpaste (Figure 5; Paper II). It was assumed that the buffering properties observed for EB@TiO_2 confirmed that mechanochemical technique does not negatively impact on the calcium carbonate structure in the eggshell (Paper II). However, the limitation noted in the study was that the protective characteristic of EB@TiO_2 alone was not compared with OTC toothpaste. This constraint was due to the fact that the gas test technique required a model that contains eggshell.

Acknowledging the limitation on the experimental design reported in Paper II, the acidic resistance of EB@TiO_2 was evaluated using a bovine model (Paper III and IV). The use of a bovine model to study enamel erosion has been widely recommended in the literature owing to the similarities with the human tooth (Yassen, Platt and Hara 2011). The acid-resistant properties of EB@TiO_2 were evaluated in situ in comparison with some commercial desensitising agents (Table 1, Paper III). In contrast to the pressure sensor technique, a more conventional technique such as FESEM, AFM, and Raman was used to evaluate the demineralisation of the tooth exposed to a solution containing HCI and the samples (Paper IV). Baseline evaluation of the mean square roughness (R_{rms}) was done on the tooth before acid exposure using AFM. The prepared specimens were randomly assigned to different groups while tooth specimen exposed to HCI alone serves as the control (Paper IV).

Paper IV established that the unexposed tooth samples and the samples exposed to HCI alone were significantly difference. On the contrary, the Bonferroni test failed to show any statistical different between the unexposed tooth samples and those exposed in toothpaste and EB@TiO_2. Nonetheless, it was established that the protective effects of EB@TiO_2 were superior to the tested toothpaste (Table 2; Paper IV). Equally significant, the FESEM and Raman test further confirmed that EB@TiO_2 offer better protection on the tooth enamel (Paper III and IV). Based on the study finding, the tested hypothesis was accepted as EB@TiO_2 offer more protection against erosive acids than commercial toothpaste.

5.2 Research hypothesis two: Evaluating the remineralization and dentin tubule occluding capabilities of EB@TiO_2

The underlining assumption for the management of DH is premised on the hydrodynamic theory that was theorised by Brännström and his co-authors over 40 years ago (Brännström, Lindén and Åström 1967). The convincing explanation suggests that external stimuli cause the movement of fluid along the dentin tubules which subsequently activates the terminals of the pulp, thereby causing pain (Brännström, Lindén and Åström 1967). As such, the ideal treatment of DH would be the use of material to obstruct or occlude the dentin tubules (de Melo Alencar *et al.* 2019). Sykes (2007) theorised that the occlusion of dentin tubules would render the movement of intratubular flows through mechanical principle. Paper V and VI assesses the occluding capabilities of EB@TiO_2, and eggshell alone in comparison with other desensitising toothpaste.

The EB@TiO_2 was prepared using the same method described above. Bovine tooth samples were used as a model to study the occluding capabilities of the composite (Paper V and VI). The sensitive model was simulated by exposing the prepared bovine dentin disc in a solution of citric acids. The discs were randomly assigned to the sample groups (Paper V and VI). A novel experimental technique for the in vitro treatment of DH was introduced. The technique involved agitation for 3 hours, using 1g of the samples in a 40mL water. The underpinning motive for the agitation technique was to mimic the protocol of using mouthwash.

Paper V uses SEM and EDX to study the occluding capabilities of the samples pre and post acidic exposure. The use of SEM and EDX were in line with the techniques reported in the literature. The SEM visibly confirm that there was complete remineralisation of the dentin tubules in the samples treated with EB@TiO_2. At higher magnification, the particles of EB@TiO_2 were very much evident. The EDX spectrum reveals that the Ti peaks observe before and after post acidic treatment were comparable (Figure 8; Paper V). However, the limitation of the study finding in paper V is that the results were more qualitative in nature.

In paper VI, the occluding capabilities of EB@TiO_2 were assessed using SEM and ImageJ analysis. The occluded area ratio in percentage was calculated using ImageJ software. The statistical data revealed that the dentin tubule occluding capabilities were superior to that measured for eggshell alone and commercial tested desensitising agents (Table 2; Paper VI). Further to this, the effectiveness of the occluded tubules was assessed post-treatment using citric acid. The motive for this was to evaluate the acidic resistance of the tubules occluded with the EB@TiO_2 particles. Paper VI established that occlusion of EB@TiO_2 was highly effective in an acidic environment, as occluded tubules remained intact post-acidic treatment. The result further supports the findings reported in Paper II-IV.

Despite the outstanding occluding capabilities of EB@TiO_2 reported in Paper V and VI, notable constraints emerging from Paper V and VI is that the compared desensitising paste were more of a nerve depolarising agents than occluding agents. Equally, the agitation test, although very much effective to study dentin tubule occlusion in a laboratory setting, it, however, has some limitation for clinical application. Traditionally, tooth brushing using toothpaste is a common method of oral hygiene worldwide (Hara and Turssi 2017).

Given the above-mentioned drawbacks, Manuscript I assesses the occluding capabilities of EB@TiO_2 in comparison with a known occluding desensitising agents (Pro-Argin and NovaMin) that have been clinically proven to some extent (West, Seong and Davies 2015). A seven-day brushing protocols (morning and night) was followed. Brushing was carried using prepared bovine dentin discs, and an automatic toothbrush for a 2min contact time. The treatment condition employed entails treating without saliva and treating with saliva (Manuscript I). The motive for the research design is influenced by the clinical condition where some patients are subject to hyposalivation and Xerostomia that reduces or limit their salivary flow (Neel *et al.* 2016). As reported in the literature (Kleinberg 2002; Merh *et al.* 2015), saliva facilitate the remineralisation of dentin tubules, by serving as a medium to deposits calcium and phosphate ions. This concept of the saliva action was also behind the technology for the development of the Pro-argin that uses calcium carbonate and arginine which mimic the natural effect of saliva in aiding tooth remineralisation (Kleinberg 2002). The same observation technique using SEM and ImageJ software described in manuscript VI was used to measure the occluding capabilities of the tested samples each day after brushing.

Manuscript I established that as the brushing days increase the remineralisation or dentin tubule occluded by each respective desensitising agents improved. It was also established that the occluding capabilities of EB@TiO_2 were more superior to both Pro-argin and NovaMin products in both saliva and without saliva. The occluding properties of EB@TiO_2 was attributed to the high availability of calcium together with the inherent properties of titanium dioxide nanoparticles. More so, it was establish in the Manuscript I that bovine dentin samples treated with Pro-argin without saliva were by far superior to those treated with saliva. It was assumed that the artificial saliva used might have contributed to the inferior tubule occlusion.

On the hand, the dentin tubules observed for the samples treated NovaMin containing the desensitising agent showed it was more effective with saliva when compared against saliva (Manuscript I). The inferior dentin tubule occlusion observe for the NOvaMin treated samples without saliva was attributed to the trapping of Ca^{+} and PO_4^{3-} ions in the bioglass. On the Contrary, the better tubule occlusion observe for the samples treated with saliva was attributed to the ability of the saliva to facilitate the deposition of Ca^{+} and PO_4^{3-} ions into the tubule, thus contributing to the gradual obstruction of the dentin tubules (Manuscript I).

Equally important, the effectiveness of the occluded tubule from each respective samples were exposure after the seven-day brushing in 4% citric acid. Manuscript I confirmed that the specimens treated with EB@TiO_2 remained intact post acidic exposure with or without saliva. However, while the samples treated with NovaMin showed improved acid resistant with saliva, its effectiveness was notice to diminish in the samples treated without saliva. The reverse scenario was observed for the Pro-argin samples, as the specimens treated without saliva shows better acidic resistance. In light of the study data reported in Paper V, VI, and Manuscript I, the tested hypothesis was rejected as there was no significant difference observed in the dentin tubules occlusion pre-and post-acidic exposure.

Part of the inquiry of this study was to develop a mathematical model to predict the remineralisation of desensitising paste. Manuscript II described the use of the logistic equation to predict the remineralisation of the samples. The input parameter used for the model development was based on the experimental data reported in Manuscript I while MATLAB Least-square fitting routine fmincon in the optimisation toolbox was used to construct the model. The motive for the use of the logistic equation was premised on the high validation and application in many fields of study (Alt and Markov 2012; Bersani and Dell'Acqua 2012; Markov 2014). The condition used for the model descriptor was based on three parameters, namely; time (t), amount of calcium or phosphate deposition, and the size (s) of the dentin tubules.

Manuscript II established that the logistic equation effectively predicted the remineralisation trends of EB@TiO_2 and Pro-argin toothpaste (Colgate Pro-relief). However, the trajectory graph obtained for the NovaMin (Sensodyne repair) had a poor fitting to the model (Figure 1; Manuscript II). It was proposed as a future study, the development of a more complex model to better understand the reminerisation trends of the product with or without saliva.

5.3 Research hypothesis three: Assessing the abrasion rate of EB@TiO_2 and commercial OTC toothpaste

Although toothpaste are traditional vehicles to deliver desensitising agents (Cummins and Marsh 2018), this may become counterproductive if the abrasives in the toothpaste are very abrasive. It has been suggested in the literature that the abrasives in the toothpaste are the main culprit in most dental problem which may likely reopen the occluded dentin tubules during tooth brushing procedure (Arnold *et al.* 2016; Rath *et al.* 2016). Manuscript III assesses the abrasivity of EB@TiO_2 in comparison with calcium carbonate, and hydrated silica containing toothpaste. Bovine enamel specimen was used for the in vitro experiment. The prepared specimens were randomly assigned to different groups (Table 1; Manuscript III). Brushing protocol was simulated in the laboratory using automatic tooth brushing, and a load of 200g. Brushing was performed at room temperature using 100mg of the respective samples at a contact time of 2min.

Since clinical evaluation appears to be impossible, laboratory procedures are acknowledged as the only method to assess the abrasivity of toothpaste (Hara and Turssi 2017). The abrasiveness of each sample was assessed using both AFM and SEM. The height difference of the brushed and covered halves of the tooth was measured using AFM, and the mean surface roughness (R_a) used for statistical analysis. In addition, and complementing the R_a values, AFM 3D images (Figure 2; Manuscript III) and SEM (Figure 3; Manuscript III) were used to provide more descriptive virtualisation of the brushed and covered surface.

Manuscript III established that enamel loss from the brushed surface, regardless of the sample group, were statistically different when compared to the covered surface. Notably, the abrasivity of EB@TiO_2 were comparable with the calcium carbonate toothpaste (Table 2; Manuscript III). Given that titanium dioxide is considered highly abrasive (Harris 1999), it was assumed that the modification of eggshell with titanium dioxide did not negatively impact on the carbonate structure. Moreover, the calcium carbonate containing toothpaste was less abrasive than the hydrated silica (Table 2; Manuscript III). Although silica and calcium carbonate has a similar chemical composition, the higher abrasiveness measured in the silica products were attributed to the complexity of the different materials found in the paste. In light of the study findings, the null hypothesis was accepted as the abrasivity of EB@TiO_2 was not statistically different from other tested OTC toothpaste.

In conclusion, the critical review has extensively shown the rigor and demonstrate the depth of the study. In particular, the experimental finding has provided evidence on the suitability of EB@TiO_2 as an active ingredient in toothpaste formulation. Ultimately, and bearing in mind the desire to address the inequality gap with respect to oral healthcare, a EB@TiO_2 desensitising toothpaste could possibly empower patients in achieving a holistic oral health which is high on the global agenda on preventive healthcare. The next chapter provides the conclusions drawn from this study. This will include the recommendations made, which will steer the study for future research.

Chapter Six – Conclusion and Recommendations

Dentin hypersensitivity (DH) constitute a public health concern and a potent challenge for oral healthcare providers to effectively manage. While there are yet to be established a gold standard technique for a long term treatment, the focus of this study was to evaluate in vitro a new approach using eggshells (a natural waste material), and titanium dioxide (EB@TiO_2) for DH management. A quantitative research approach and an experimental research design that consists of three phases were adopted. In phase one of this study, different characterisation techniques were used to confirm the modification of EB@TiO_2. Phase two, on the other hand, assesses the suitability of the EB@TiO_2 as an oral healthcare product by examining its cytotoxicity and antibacterial properties. By contrast, phase three investigated the quality of the EB@TiO_2 as a new approach to the management of DH. Particularly, the acid resistant, abrasivity, and remineralisation characteristics of EB@TiO_2 were studied. This chapter concludes by drawing on the discussion of the above-mentioned phases to provide recommendations by proposing directions for future research.

6.1 Revisiting the research objectives

Objective One: was to develop and characterise modified Nano-sized Eggshell-Titanium oxide (EB@TiO_2) desensitising agents.

The sub-objectives are:

1.4.1 To determine the physical characteristics of EB@TiO_2 desensitising agents in terms of crystallisation; particle size; and phase, and shape in order to establish the bond strength characteristics of the newly developed EB@TiO_2 desensitising agents.

The findings of this study have explicitly underscored the different characterisation methods (FTIR; XRD; EDX; SEM, and HRTEM) to show that eggshell powder and titanium dioxide was successfully modified via the mechanochemical method by ball-milling application (Paper III and IV). Importantly, it was confirmed that the ball-milling process do not cause phase transformation, and thus, did not negatively impact on the calcite composition of the eggshell (Paper III). Additionally, the image J analysis reveals that EB@TiO_2 was in a nanometer range (Paper IV).

1.4.2 To conduct thermal analyses of EB@TiO_2 desensitising agents using thermo-gravimetric analysis (TGA) in order to determine the thermal stability and degradation of the product.

The TGA degradation curve confirms that EB@TiO_2 composition has high thermal stability, and thus makes it suitable and economically viable as a desensitisting agent (Figure 1: Addendum 1).

1.4.3 To determine the chemical constituents of EB@TiO_2 desensitising agents using Energy Dispersive spectroscopy (EDX) to ensure that product is free of hazardous substances.

SEM and HRTEM analysis further confirmed that the TiO_2 was spread on the surface of the eggshell powder (Paper IV). More so, the EDX reveals Calcium, Oxygen, carbon, and Titanium to be the predominant elements found in the EB@TiO_2. Another trace element found in the composite was Magnesium (Paper IV). The aforesaid chemical elements confirm that the composite is free from a hazardous, and heavy metal substance.

Objective Two: To determine biocompatibility and conduct microbial assessment of Titanium oxide and Eggshell base (EB@TiO_2) desensitising agents.

The sub-objectives are:

1.4.5 Using a Bacteria strain *E.coli* (ATC 25922 strain) and *B. Cereus* (ATC 10876) to assess the antibacterial properties of EB@TiO_2 desensitising agent in line with ISO 21149 and ISO 16212 for cosmetic products.

The antibacterial assessment of EB@TiO_2 revealed that the product exhibit inhibitory properties against *E.coli,* and *B. Cereus* bacterial strain (Table 2 Addendum 2).

1.4.6 Using NIH 313 and BHK21 cell lines, the toxicological profile of EB@TiO_2 desensitising toothpaste will be characterize to ascertain its cytotoxicity in line with ADA 1979 standards.

In terms of the safety of the product, the prominent aspects of this study confirm that EB@TiO_2 showed little effect on both cell lines even at the highest concentration of 100µg/ml (Paper V and Manuscript III).

Objective Three: To establish the product-based quality of Titanium oxide and Eggshell base (EB@TiO_2) in terms of 'fitness for purpose'

The sub-objectives are:

1.4.7 To determine the abrasivity of EB@TiO_2 desensitising toothpaste on bovine enamel in line with recommended standard by American Dental Association (ADA) and the International Standard Organisation (ISO).

In terms of the abrasiveness of the EB@TiO_2 on the bovine enamel, this study found that the abrasive wear rate of EB@TiO_2 was comparable to other toothpaste products containing calcium carbonate (Manuscript I). This strongly reaffirm the suitability of EB@TiO_2 for use as an oral care product.

1.4.8 To evaluate the acid resistant properties of EB@TiO_2 using a scanning electron microscope (SEM), and atomic force microscope (AFM), Raman spectroscopy, and pressure sensor in order to determine its suitability for enamel remineralisation.

This study conclusively showed that the EB@TiO_2 offer better protective covering to the bovine enamel against acid attack (paper II, III, and IV). This further confirmed that EB@TiO_2 is a suitable material for enamel remineralisation.

1.4.9 To examine the effectiveness of EB@TiO_2 desensitising toothpaste in occluding open dentine tubules using a scanning electron microscope (SEM) and ImageJ software.

Equally important, this study confirms that the modified EB@TiO_2 composite effectively occlude dentin tubules (Paper V and VI).

1.4.10 To evaluate the occluding characteristic of EB@TiO_2 in reducing DH compared with commercial toothpaste containing Pro-Argin and NovaMin with or without saliva.

This study confirmed that EB@TiO_2 occluding capabilities were superior when compared with a commercial toothpaste containing Pro-Argin and NovaMin with or without saliva (Manuscript III).

1.4.11 To use a mathematical equation to forecast the duration of dentin tubule remineralisation on bovine teeth treated with EB@TiO_2 desensitising toothpaste.

This study reveals that the dentin tubule occlusion was effective after 3 days of brushing (Manuscript II). This is in line with achieving objective 1.4.11, which is to use a mathematical equation to forecast the duration of dentin tubule remineralisation on bovine teeth treated with EB@TiO_2 desensitising toothpaste.

Thus the aim of the study, namely, to develop and evaluate *in vitro* the quality of a modified nano-sized eggshell and titanium dioxide (EB@TiO_2) composite as the active ingredients in the management of DH was achieved as illustrated by the study findings. The study hypotheses were extensively elaborated in the developed manuscripts (presented in Chapter 5).

6.2 Limitation

Although the cytotoxicity assay conducted suggest that EB@TiO_2 had no adverse effects on the cell lines tested, a vivo research is, however, needed to fully and comprehensively characterize the cytotoxicity before its potential commercialisation.

6.3 Recommendations

- Despite the outstanding dentin tubule occluding and acid resistant properties of EB@TiO_2, it is highly recommended that caution be applied for its use in oral care product. This is in line with the view that nanotechnology, particularly in Africa is still emerging and little is known about its safe use in cosmetic and personal care product.
- Additionally, a more robust laboratory test such as hydraulic conductance would help in understanding the occluding characteristics of EB@TiO_2.

- Further to the above, and given that the sensation to pain is subjective and transient, it is highly important that clinical study of EB@TiO_2 in reducing DH be conducted.

- The above studies would help strengthen and establish the suitability of EB@TiO_2 as a new approach for the management of DH. This supports future investigations into the clinical trials of EB@TiO_2.

In summary, this study has exhaustively demonstrate that a modified nano-sized eggshell waste and titanium dioxide is an effective strategy in the overall management of DH. The result confirm that the composite exhibit an outstanding acid resistance characteristics which is essential in the maintenance of oral health. The study therefore provide a new evidence and approach for the management of DH, particularly in low income countries were cost of oral healthcare may be too high.

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Addendum 1: Thermogravimetric analysis

From Figure 1 the TGA curves show the incidence of two thermal events within the temperature range of 50-900ºC. The first phase (600ºC) is attributed to the decomposition of the anatase form of titanium dioxide, which caused a small weight loss (~ 1.061%). The second phase (699.14ºC) is endothermic, and is linked to the decomposition of calcium carbonate to carbon dioxide and calcium oxide (Chaudhuri B *et al.* 2013). This weight loss equated to approximately 30.41% of the total mass.

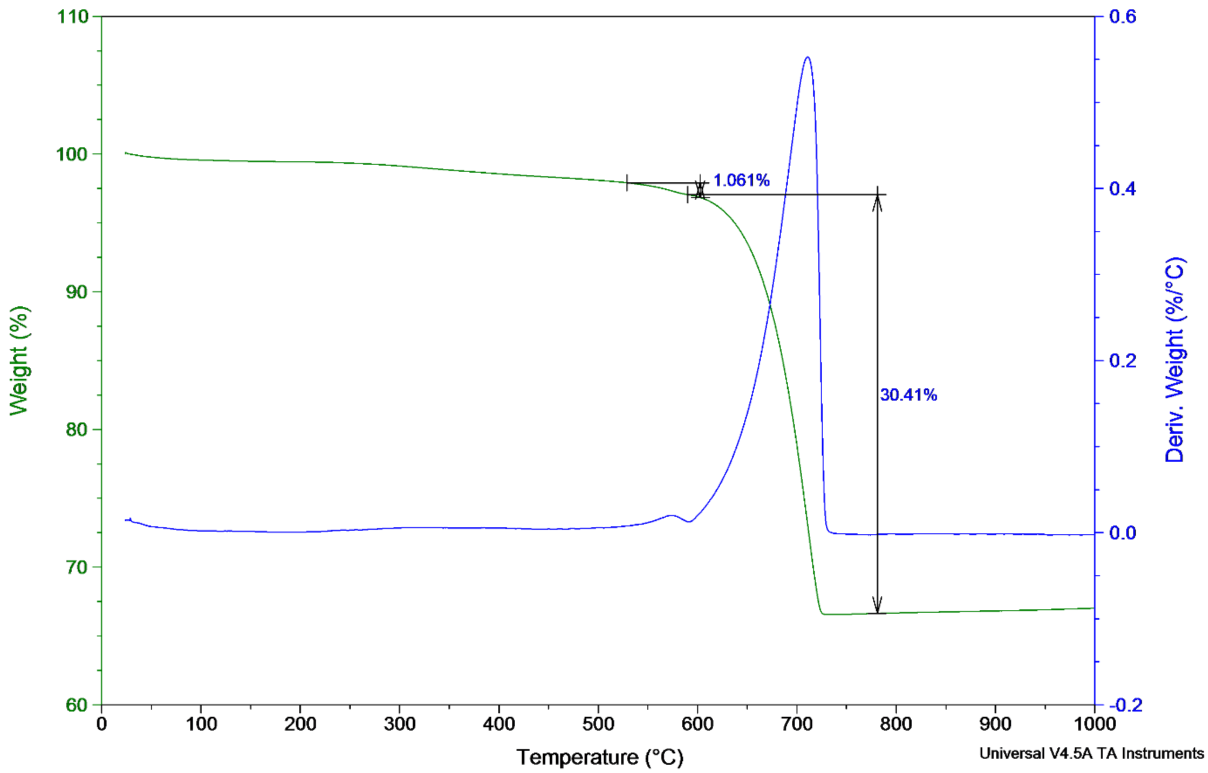

Figure 1: TGA curves of EB@TiO_2.

It is worth mentioning that the above result was presented and published as a conference paper.

Onwubu, S.C., Mdluli, P.S. and Singh, S. 2018. The remineralization potential of a modified eggshell-titanium composite. In: proceeding of the 3rd International conference of composite and biocomposite, Port-Elizabeth, 57

Addendum 2: Antibacterial properties

The antibacterial screening of the EB@TiO_2 compounds at different ratio of eggshell and titanium dioxide modification (2:1, 3: 1, and 4:1) was determine using the disc diffusion method against *E. coli* and *B. Cereus*. The results obtained have shown that these compounds displayed a slight inhibition against *E. coli* and *B. Cereus* (Table 1). It was also found that irrespective of the ratio of eggshell and titanium dioxide used, the inhibitory properties observed for EB@TiO_2 were more or less the same. On the contrary, tested toothpaste (Colgate Pro-relief, Sensodyne Repair, Sensodyne rapid relief), and eggshell alone didn`t display any inhibition against the bacteria used.

Table 1: Inhibitory properties of the samples

Compounds	***E. coli*** **25922 (mm)**	***B. Cereus*** **10876 (mm)**
EB@TiO_2 (2:1)	6.1±0.5	6.0±0.0
EB@TiO_2 (3:1)	6.0±0.0	6.0±0.0
EB@TiO_2 (4:1)	6.0±0.0	6.0.±0.0
Eggshell	0.0±0.0	0.0±0.0
Titanium dioxide	6.0±0.0	7±0.0
Colgate Pro-relief	0.0±0.0	0.0±0.0
Sensodyne repair	0.0±0.0	0.0±0.0
Sensodyne rapid relief	0.0±0.0	0.0±0.0
Ciprofloxacin (Control)	18.6±0.5	22.5±1.0

The inhibitory zone observed for EB@TiO_2 at different ratio composition are given in Figure 2. The image visibly confirmed the inhibitory properties of EB@TiO2 against *E. coli* and *B. Cereus*.

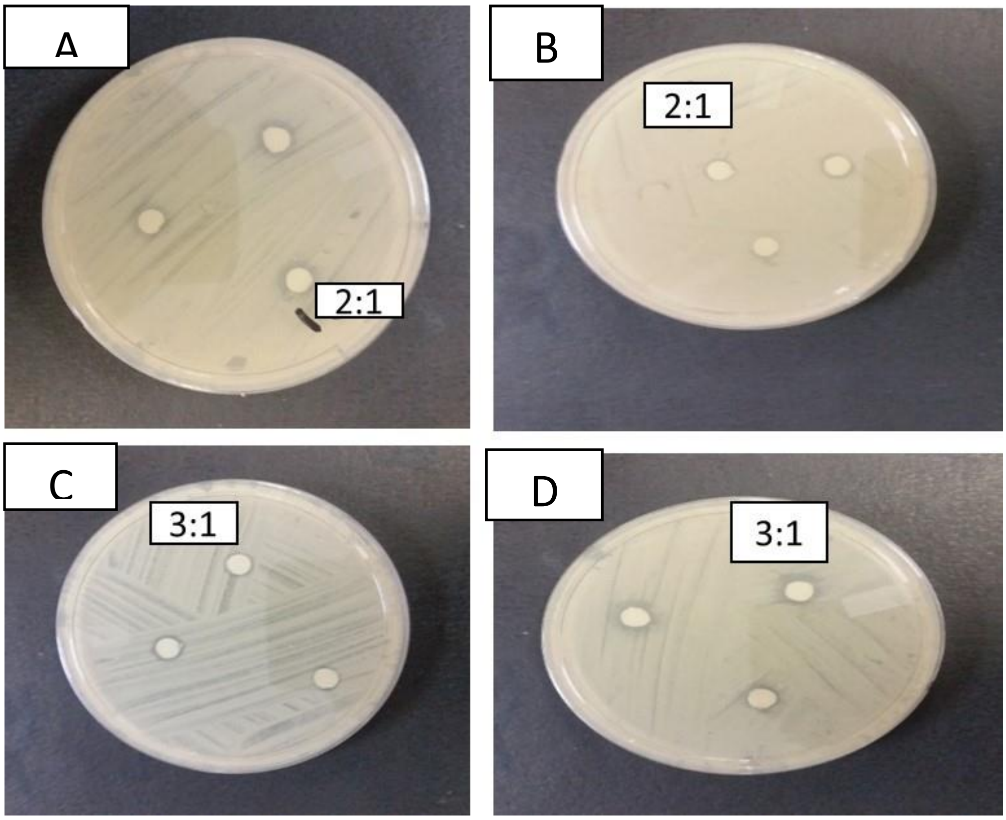

Figure 2: Inhibitory zone (mm) of compounds 2:1 against *E. coli* (A) and compounds 3:1 against *B. Cereus* (B), Compounds 3:1 against *E. coli* (C), compound 3:1 against B (D) against *B. cereus*