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A comparative study of mesoporous nanohydroxyapatite bionanocomposite from eggshells and fish scales through a mechanochemical method

Submitted in fulfilment of requirements of the degree of
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ABSTRACT

Dental hypersensitivity [DH] is a common pain amongst dental patients with a high negative impact on the quality of life. The onset of DH is attributable to the demineralisation of tooth enamel from excessive consumption of acidic drinks and beverages. While different materials have been reported for DH management, the application of nanoparticles is suggested to be the future of DH management. Particularly, the remarkable properties of mesoporous silica and nanohydroxyapatite have promoted their use for DH management. These materials are often synthesized following the wet-chemical route. However, the use of toxic chemicals and the cost associated made these processes infeasible for DH management. Given the said concern, this study synthesises nanohydroxyapatite (nHAp) from eggshell and fish scale waste and modified it with mesoporous silica to create a bionanocomposite (MSN@nHAp) via the mechanochemical method. Part of the study inquiry was to comparatively assess the remineralisation characteristics of nano-hydroxyapatite (nHAp) extracted from waste eggshells and fish scales.

A quantitative approach to experimental research design is adopted in this study which includes three phases. The first looks to develop nanocomposite using nano-sized hydroxyapatite and mesoporous silica, then characterise the nanocomposite formed. Phase identification of the crystals was confirmed by Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) applied to determine particle size and morphology. The second assesses the quality of the nanocomposite, its biocompatibility, and its toxicological characteristics. The third evaluates the remineralization capabilities of nHAp in occluding open dentin tubules. The mean values of the occluded area ratio in the SEM study were evaluated with one-way analysis of variance (ANOVA) with statistical software (IBM SPSS Statistics v28; IBM Corp), followed by a multi-comparison test with Bonferroni correction ($\alpha = .05$). The validity of the study was achieved following SANS 1302 (2008) requirement for preparation, developing, and testing toothpaste. The reliability was determined via the reproducibility and repeatability of tests.

Paper 1 investigated the remineralization and acid-resistant characteristics of nanohydroxyapatite produced from eggshell waste via mechanochemistry. Paper 1 established that nHAp was successfully produced from eggshell waste after 5 hr of milling. It was found that the produced nHAp (EnHAp) was effective in neutralizing common dietary acids. the nHAp showed complete occlusion of the dentin tubules.

Manuscript 1 was based on the comparative assessment of the remineralization characteristics of Nano-hydroxyapatite extracted from fish scales and eggshells. Manuscript 1 established that nHAp extracted from eggshells (EnHAp) showed superior dentin tubule occluding characteristics than those of fish scales (FnHAp and mFnHAp). It was found that there were slight variations in physicochemical characteristics such as the Ca/P ratio, crystallinity, particle sizes and surface morphology of the nHAp extracted.

Paper II reports on the invitro assessment of the acid resistance characteristics of mesoporous silica/nanohydroxyapatite (MSN@nHAp) biocomposite synthesised through the mechanochemical method. Paper II established that the MSN@nHAp exhibits superior acid resistance characteristics.

In conclusion, the provided evidence shows that the mechanochemical method is a useful technique in the synthesis and surface modification of valuable biomaterials. The experimental finding provides a benchmark for further advance studies on utilizing natural nHAp in dentistry.

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CONFERENCE PAPER AND ABSTRACTS ARISING FROM THIS STUDY

Stanley C Onwubu, **Sandile Cromwell Mkhize**, Thabang, Hendrica Mokhothu, and Phumlane Mdluli. 2022. Acid Resistance Characteristics of Nanohydroxyapatite/Silica Biocomposite Synthesized Using Mechanochemistry. In: the 4th International Conference on Graphene and Novel Nanomaterials (GNN 2022). September 16-19, 2022, Shenzhen, China, Microsoft Teams.

Sandile Cromwell Mkhize, Stanley C Onwubu, and Phumlane Mdluli. 2019. A clean strategy for the preparation of hydroxyapatite/mesoporous bionanocomposite through a mechanical method. In: the 4th Interdisciplinary Research and Innovation Conference, Durban, South Africa.

JOURNAL PAPERS PUBLISHED AND SUBMITTED ARISING FROM THIS STUDY

Onwubu, S.C., Naidoo, D., **Mkhize, S.C.,** Mabaso, N.L.N., Mdluli, P.S. and Thakur, S. 2020. An investigation in the remineralization and acid resistant characteristics of nanohydroxyapatite produced from eggshell waste via mechanochemistry. *Journal of Applied Biomaterials & Functional Materials*, 19 (7): 1-8**.** https://doi.org/10.1177/2280800020968352

Mkhize, S.C., Onwubu, S.C., Mlambo, M. and Mdluli, P.S. 2021. An In vitro Assessment of the Acid resistance characteristics of nanohydroxyapatite/silica Biocomposite Synthesized Using mechanochemistry. *Journal of Nanomaterials*, 2021 https://doi.org/10.1155/2021/4438100

Mkhize, S.C., Onwubu, S.C., Mokhothu, T.H., Mdluli, P.S and Bisetty, K. 2021. Comparative assessment of the remineralization characteristics of Nano-hydroxyapatite extracted from fish scales and eggshells. *Journal of Applied Biomaterials & Functional Materials* ***(Under-review)***

BOOK CHAPTERS PUBLISHED ARISING FROM THIS STUDY

Onwubu, S.C., Mdluli, P.S., Singh, S., Thakur, S. and **Mkhize, S.C**., 2021. Cytotoxicity of bionanocomposites in the treatment of dentine hypersensitivity. In *Bionanocomposites in Tissue Engineering and Regenerative Medicine* (pp. 549-564). Woodhead Publishing.

Sandile Cromwell Mkhize

DEDICATION

I dedicate this work to my parents in appreciation of their unwavering support of my academic endeavours. I will never forget their unwavering love, dedication, and commitment.

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Acronyms

ANSI/ADA	American National Standards/American Dental Association
DH	Dentin Hypersensitivity
DUT	Durban University of Technology
EDX	Energy Dispersive X-ray Spectroscopy
EnHAp	Eggshell nanohydroxyapatite
FnHAp	Fish scales nanohydroxyapatite
FTIR	Fourier Transform Infrared Spectroscopy
HRTEM	High Resolution Transmission Electron Microscope
IUPAC	International Union of Pure and applied Chemistry
ISO	International Standard Organisation
mFnHAp	Milled fish scales nanohydroxyapatite
MSN	Mesoporous silica
nHAp	Nano-hydroxyapatite
RPM	Revolution per Minute
FSEM	Field- Scanning Electron Microscopy
SPSS	Statistical Package for Social Science
XRD	X-ray Diffraction

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CHAPTER ONE - INTRODUCTION

1.1 BACKGROUND AND CONTEXT OF THE STUDY

In recent times, the use of bio-based materials has gained popularity and has thus attracted several researchers that aim to explore their potential for biomedical applications (Antunes *et al.* 2019; Battegazzore *et al.* 2019). The unique properties of these materials such as their non-toxicity, abundant availability, biocompatibility, and biodegradability, and physical, chemical, and structural properties favour their use in various applications including antimicrobial coatings (Kalyoncuoglu *et al.* 2015), drug carriers (Aguilar *et al.* 2019), adsorbents (Tavakolian *et al.* 2020). Particularly, the healthcare sector has been revolutionised by the use of bio-based materials due to the growing uses in a variety of multidisciplinary fields including dentistry (Anju *et al.* 2020). From a dentistry context, the use of bio-based materials has brought about tremendous growth and advantage in dental practice. For example, bio-based materials such as hydroxyapatite have been widely used in preventive and conservative dentistry (Suresh *et al.* 2018; Vano *et al.* 2018), and restorative and periodontal treatment (Kijartorn *et al.* 2022). This, thus, makes hydroxyapatite bio-based materials a game-changer in the treatment and management of dental and oral-facial conditions.

Particularly, in the era of preventive dentistry, it is speculated in the literature that due to periodontal therapy and home care practices, more individuals will keep theirs into a later age; which might result in a greater number of exposed dentin surfaces (Balcheva *et al.* 2017). This may consequently result in a dental condition known as dentin hypersensitivity (DH). DH is a common oral health problem faced in dentistry and defined by the Canadian Association of Dentistry as a "short, sharp pain arising from exposed dentin in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other form of dental defect or pathology" (Cartwright 2014).

From an epidemiological perspective, DH is a common oral disease that affects nearly 43% of the adult population worldwide (Saeki *et al.* 2016; Onwubu *et al.* 2019c) and thus poses a challenge for an oral care provider to effectively manage. Most affected areas are buccal cervical areas of permanent teeth, followed in descending order by canines, first premolars, incisors, second premolars and molars (Balcheva *et al.* 2017). Most sufferers range in age from 20 to 40 years but the peak is at the end of the third decade (Balcheva *et al.* 2017). DH thus constitute a dental public concern as it negatively affects an individual's quality of life by restricting their daily activities (Bekes and Hirsch 2013).

Since the negative impact of DH on an individual's quality of life motivates them to seek oral care assistance (Pashley 2013), it thus means that reducing DH improves the quality of life (Douglas-de-Oliveira *et al.* 2018). While several theories have been proposed in the literature that aims to demystify the pain associated with DH (Mantzourani and Sharma 2013), the hydrodynamic theory seems to be the most widely accepted explanation by the dental community. This seminal theory by Brännström suggests that the movement of fluid inside dentin tubules stimulates the nerve, thereby causing pain (Brännström 1986). It is therefore acceptable to say that blocking the open dentin tubules will invariably control the hydrodynamic process (Sykes 2007; Onwubu *et al.* 2019a). According to de Melo Alencar *et al.* (2019), the ideal strategy in the management of DH would be to effectively occlude the dentinal tubules to prevent fluid flow. It is therefore acceptable to say that blocking the open dentin tubules will invariably control the hydrodynamic process.

In an attempt to manage DH, dentin occluding tubules materials in the form of topical desensitising toothpaste containing materials such as potassium oxalates (Cunha-Cruz *et al.* 2011), strontium salts (Saeki *et al.* 2016), sodium fluoride (Pandit, Gupta and Bansal 2012) among others are in the market. However, dentin tubules occluded by some of these materials are superficial with limited infiltration depth, showing poor acid resistant (Arnold, Prange and Naumova 2015). Given these drawbacks, a novel approach to the management of DH using various combinations of nanoparticles has been advocated in the literature (Tian *et al.* 2014; Onwubu *et al.* 2019b). Among these materials, mesoporous silica (MSN) and nano hydroxyapatites (nHAp) are projected to revolutionise the treatment of DH (Yu *et al.* 2016). The use of hydroxyapatite particles, particularly in the nano-size has emerged as a promising material for remineralizing dentin (Onwubu, Mhlungu and Mdluli 2019). This is supported by clinical evidence where nano-hydroxyapatite (nHAp) based desensitising agents have shown promising clinical improvements in DH (Suresh *et al.* 2018; Vano *et al.* 2018).

While previous studies have extracted nHAp from various bio-waste such as sea shells (Santhosh and Prabu 2013), porcine teeth and bones, eggshells, and bovine bones (Brzezińska-Miecznik *et al.* 2015; Irfan *et al.* 2020; Onwubu *et al.* 2020b), there is limited evidence in the use of fish scales, particularly in the extraction of nHAp for the management of DH. Studies have shown that fish scales contain numerous valuable inorganic and organic such as collagen

(40-55%) and hydroxyapatite (15%) which makes a useful alternative for the extraction of hydroxyapatite (Boaventura *et al.* 2020; Kulkarni and Maniyar 2020). As such, fish scale waste has tremendous but unexploited biomaterial that could potentially add value to waste.

Furthermore, mesoporous silica (MSNs) has been widely used as a dentin tubule occluding material in combination with other nanohydroxyapatite. Tian *et al.* (2014) showed that a new MSN-based biocomposite successfully occludes dentin tubules. Yu *et al.* (2016) reported that the mesoporous silica/hydroxyapatite composite was effective against acidic substances in occluding dentin tubules. While MSN@nHAp biocomposites are widely synthesized in the laboratory through the precipitation process (Zhou and Lee 2011), nevertheless, the chemical method of synthesizing the composite may be cumbersome and time-consuming and, most times requires the use of toxic chemicals (Googerdchian *et al.* 2018). Consequently, there is a need for an alternative environmentally friendly technique for synthesizing the biocomposite using the mechanochemical method. Moreover, the application of the mechanochemical method in biocomposite material preparation has gained widespread interest among researchers, due to its simplicity and environmental friendliness (Baláž 2018). The technique utilises mechanical force to affect chemical reactions as well as structural changes in a material (Hua *et al.* 2017).

While the mechanochemical method has great potential in material preparation, there is limited evidence of its use for the preparation of mesoporous silica and nanohydroxyapatite biocomposites (MSN@nHAp). There is also a dearth of research evidence for its application in extraction. The purpose of this study was to synthesize and extract nanohydroxyapatite from waste eggshells and fish scales. Modified the extracted nHAp with mesoporous silica by the application of a mechanochemical method for potential use in dentistry. Part of the study enquiry is to provide a benchmark for further advanced studies on utilising natural nHAp in remineralizing dentin.

1.2 RESEARCH PROBLEM

Over the last decades, synthetic materials had served many needs of human endeavour ranging from engineering, medicine, and the food industry. However, with the recent concern on the impact's synthetic materials, particularly the effect petrochemical products have on the environment, researchers, as well as corporate organizations, are now paying keen interest in sustainable materials for the environment (Shit and Shah 2014). Studies have shown that there is a significant shift from synthetic materials to bio-based materials in a variety of multidisciplinary fields including dentistry (Liu *et al.* 2019). From a dentistry context, the use of bio-based materials in their nano-size forms has brought about tremendous growth and advantage in dental practice. Nanomaterials such as hydroxyapatite and mesoporous silica have been advocated as an effective strategy in the management of DH (Onwubu, Mdluli and Singh 2018).

A study by Yu *et al.* (2016), for example, claims that the mesoporous silica-hydroxyapatite combination offers better dentin tubule occlusion with outstanding resistance to acidic attack. However, the current method of synthesising the MSN@nHAp is noted to be time-consuming and requires the use of toxic chemicals (Mondal *et al.* 2017). As a result of all these drawbacks, this study will focus on using readily available eggshells and fish scales to synthesize nanohydroxyapatite bionanocomposite (MSN@nHAp). The mechanochemical method is highly considered an environmentally friendly method with high reproducibility, consistency, flexibility and cleanliness (Baláž 2018; Onwubu, Mdluli and Singh 2019). Besides, this approach is usually quicker and cheaper than conventional wet-chemistry techniques (Baláž 2018). This is also resonating further with the proposition made by Schmidlin and Sahrmann (2012) that a non-invasive, non-hazardous, easy-to-apply material remains needed for the management of DH.

1.3 AIM

To synthesize a mesoporous nanohydroxyapatite bionanocomposite from natural waste by the application of a mechanochemical method for potential use for dentistry as a remineralizing agent.

1.4 OBJECTIVE OF THE STUDY

The research objective was achieved in three phases.

Objective One: Preparation and extraction of nHAp from bio-waste

The sub-objectives are to:

1. Extract nHAp from eggshells using calcination and mechanochemical method.
2. Synthesize mesoporous nanohydroxyapatite bionanocomposite (MSN@nHAp) from eggshells using the mechanochemical method.
3. Extract nHAp from fish scales using alkaline hydrolysis and mechanochemical method.

4. Compare the physicochemical characteristics of eggshells and fish scales nHAp using different analytical techniques

Objective Two: In vitro assessment of the cytotoxicity of nHAp extracted using mouse fibroblast cell culture.

Objective Three: Assessing in vitro the fitness of purpose of the bio-based nHAp

The sub-objectives are to:

1. Assessed the remineralisation characteristics of nHAp extracted from eggshells.
2. Comparatively assessed the remineralisation characteristics of nHAp extracted from waste eggshells and fish scales.
3. Assessment of the acid resistance characteristics of mesoporous silica/nanohydroxyapatite (MSN@nHAp) in comparison with nHAp from eggshells.

1.5 RESEARCH HYPOTHESES

1.5.1 H_0: There is no significant difference in the occluding abilities of the nHAp extracted from eggshells (EnHAp) and those extracted from fish scales (FnHAp).

H_1: There are significant differences in the occluding abilities of the nHAp extracted from eggshells and fish scales.

1.5.2 H_0: Modification of EnHAp with mesoporous silica (MSN@nHAp) had no difference in its protective effect against erosive acids.

H_1: Modifying EnHAp with mesoporous silica (MSN@nHAp) significantly improved its protection against erosive acids.

1.6 RATIONALE/SIGNIFICANCE OF THE STUDY

According to Ronan and Kannan (2017), about 7.7 million tons of eggshell waste were generated in 2014. The data provided by the Food and Agricultural Organisation (FAO) indicates that by 2020, global eggshell waste production is likely to hit 86.8 million tons (Food and Agriculture Organisation 2014). This implies that the amount of eggshell waste produced and disposed of by food processing industries may continue to increase with time. In addition to this, a huge amount of waste is currently being produced from the fish market in the form of fish scales, which are either dumped or left to decay creating severe environmental issues in the form of bacterial incubators (Aziz *et al.* 2021). Of particular concern, these wastes generate huge amounts of methane and other environmental foe gases that pose a significant challenge to the environment (Mejía-Saulés *et al.* 2006). The development of valuable sustainable products from the eggshell and fish waste would thus serve as a double purpose choice not only to gain a sustainable source of material for nHAp extraction but also the creation of a valuable product. Furthermore, using eggshell and fish scale in the extraction of nHAp is vital in the environmental problem of waste management. This will also help towards achieving a circular economy, which is high towards a cleaner and greener environment. This study, therefore, aligns with the United Nations Sustainable Development Goal (SDG13) which speaks on urgent action to combat climate change and its impacts on the environment. By developing valuable and cheaper biomaterials from waste, this study will also address part of the South Africa National development plan (NDP 2030), which aims to reduce poverty and inequality in the country.

Furthermore, this study will add towards the development of an innovative strategy in the matter of reducing DH and remineralising dentinal tubules using nHAp extracted from bio-waste. As such, and from a material perspective, the study provides a benchmark for further advanced studies on utilising natural nHAp in dentistry.

1.7 DELIMITATION/SCOPE OF THE STUDY

Although nHAp can be extracted from various bio-waste such as corals, cuttlefish shells, porcine teeth and bones, and bovine bones, this study will only be using eggshells and fish scales to extract nHAp. This is attributed to the huge availability of materials in South Africa as waste.

1.8 STRUCTURE OF THE THESIS

This study consists of five chapters:

Chapter 1: Introduction

This chapter provides a general background and provides a context for the study. This chapter also contains the problem statement, the aim, and the specific objectives of the research

Chapter 2: Literature Review

An overview of the literature on the structure of the human tooth, Tooth sensitivity and its causes and trends in the management of DH. Introduction of eggshells and fish scales, Hydroxyapatite and bionanocomposite composition, characteristics, and applications.

Chapter 3: Research Methodology and Design

Detailing the specifics of the quantitative research paradigm, parameters and experimental research design that is to be adopted in this study.

Chapter 4: Critical discussion

Provides a critical discussion of the study findings. The linkages of the published and unpublished manuscripts were presented with a view of addressing the research objectives and hypotheses.

Chapter 5: Conclusion and recommendations

Provides the conclusions in line with achieving the research objectives. In addition, the recommendations drawn from the study and future directions for the study were presented in this chapter.

A list of works cited is included after the dissertation.

1.8 SUMMARY

Chapter one has provided a review and the background to the use of bio-based materials such as nanohydroxyapatite from biowaste in dental applications. The chapter provided the rationale and motivated for the importance of extracting value-added products from bio-waste.

CHAPTER TWO – LITERATURE REVIEW

Dentin hypersensitivity (DH) is a public health concern with a severe negative effect on people's quality of life. The use of biomaterials, particularly in their nanoform, has been touted as an effective strategy in the management of DH. This chapter reviews literature related to the management of dentin hypersensitivity. The review presents an overview of the structure and characteristics of the human tooth and the aetiology of DH. Overall, this literature review is structured into five sections. Section one discusses the structure of the human tooth enamel in terms of its composition, characteristics, and properties. Section two describes the aetiology of DH. Here, the factors contributing to the onset of DH will be detailed. Section three discusses dental caries as a public health concern. Thereafter, the process of DH development and the theories supporting DH were presented and discussed. Section five reviews the literature on the management of DH. Here, the use of nanomaterials as an effective strategy for the management of DH was detailed with supporting evidence. Section six presents the source of extracting nanohydroxyapatite (nHAp). Here, the motivation and rationale for the choice of both eggshells and fish scales were elaborated upon. This chapter concludes with a discussion of the science of mechanochemistry which is an environmentally friendly technique for the extraction of nHAp from biowaste.

2.1 OVERVIEW AND STRUCTURE OF THE HUMAN TOOTH

Healy (2016) reveals that the human tooth is composed of enamel, cementum, and dentin, which form the crown of the tooth, and the pulp. The tooth enamel is the hardest material found in the human body and therefore functions as a protector for the sensitive parts of the tooth from damage and guards against wear and tear (Lynnerup and Klaus 2019). The enamel forms in an extracellular space lined by ameloblasts, which control both the ionic and the organic contents of the enamel extracellular space (Mitsiadis *et al.* 2014). Nanci (2017) notes that the ameloblasts cover the entire surface of the layer as it forms but is lost as the tooth emerges into the oral cavity. According to the author, the loss of the ameloblasts renders the enamel a nonvital and insensitive matrix that, when destroyed by any means, cannot be replaced or regenerated (Nanci 2017). Although the enamel can be regarded as dead tissue in a strict biologic sense, nonetheless, it is permeable (i.e. ionic exchange can occur between the enamel and the environment of the oral cavity) (Nanci 2017).

While the enamel cannot regenerate itself, it is, however, capable of some limited repair by physicochemical means. This is the premise of this research in attempting to use nanohydroxyapatite extracted from biowaste in the repair of damaged teeth. Wazen and Nanci (2013) revealed that in the presence of an appropriate supply of calcium and phosphate ions from the saliva remineralization of the enamel can occur in the subsurface enamel. Moreover, mature enamel consists of more than 96% inorganic material in the form of apatite crystals and traces of organic material (4%) and water (Nanci 2017). Furthermore, Field (2012) reveals the main chemical constituent of the enamel to be calcium apatite (96-98%), either as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) or fluorapatite ($Ca_{10}(PO_4)_6F_2$).

The hydroxyapatite, which forms the enamel, exhibits peculiar dimensions and organization of its crystallites. Due to the chemical composition of the enamel, other authors (Bath-Balogh and Fehrenbach 2011; John *et al.* 2015) have shown that the enamel is susceptible to demineralization. This is of particular concern as the enamel, which is the outermost layer of the crown of a tooth, protects the underlying dentin and pulp tissue from the external oral environment (Desoutter *et al.* 2014). It thus meant that the demineralisation or loss of the enamel exposes the underlying dentin to the external environment, which in turn leads to dentin hypersensitivity (Taha *et al.* 2015).

The dentin itself is a vital tissue, consisting of dentinal tubules, and is naturally sensitive because of extensions of odontoblasts and the formation of dentin–pulp complex (Miglani, Aggarwal and Ahuja 2010). Dentin is sensitive to stimuli due to the lesion extension of the odontoblastic process and the formation of the dentin-pulp complex (Miglani, Aggarwal and Ahuja 2010). The pulp is found in the centre of the tooth and contains all the blood vessels and nerves that keep the tooth alive. The pulp is extremely sensitive to any kind of trauma or damage.

Whilst the dentin and pulp are histologically different, they have the same embryonic origin; ectomesenchyme origin (Huang 2011). It is therefore reasonable to assume that the formation of dentin-pulp causes dentin to be affected by pulp and vice versa. Dentin has very minute tubules (Figure 2.1), which are filled with the odontoblastic process (Davari, Ataei and Assarzadeh 2013). Given the propensity of the dentin to affect the pulp, it thus means that exposed dentin tubules will trigger tooth sensitivity. Strong knowledge and understanding of the cause of DH and the predisposing factors are highly essential to help develop effective treatment and management strategies.

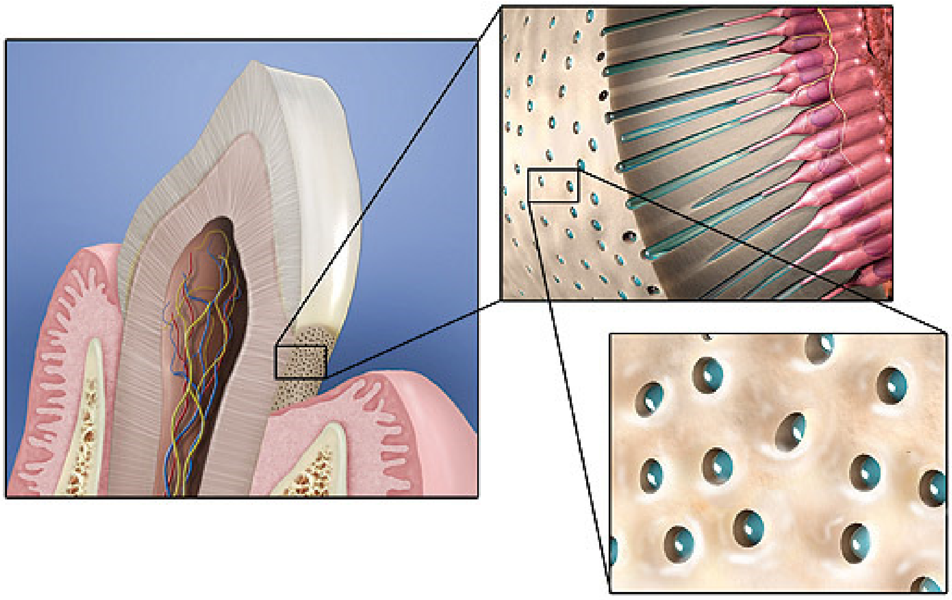


Figure 2. 1: Dentin structure showing tubules (Source (Panagakos 2011)

2.2 ETIOLOGY OF DENTIN HYPERSENSITIVITY

The aetiology of dentin hypersensitivity (DH) is poorly understood and thus requires further investigation into its management. DH is one of the most common painful conditions affecting oral comfort and function. It is also one of the least successfully resolved problems of the teeth (de Oliveira da Rosa *et al.* 2013). Under normal conditions, dentin is covered by enamel and does not suffer direct stimulation (Orsini *et al.* 2010). Dentin’s sensitivity to stimuli does not lead to any problem while it is covered with protective tissues, such as enamel, and cementum (Cummins 2010). The human oral cavity is, however, bombarded by both external and internal agents that may erode the enamel. The consequence is that this may expose the dentin to the external environment.

Although the enamel is the hardest substance developed in the human system, it is, however, subject to harsh wear, due to its role in mastication, the nature of the oral cavity and the environment. Hemingway *et al.* (2006) reported that the causes of enamel surface loss are multifactorial and, thus, rarely seen in isolation. Table 2.1 outlines the predisposing factors that could influence enamel surface. Thus, the causes of enamel surface loss, particularly from non-carious lesions include attrition, erosion, abrasion, and abfraction.

Table 2. 1**:** Multifactorial causes of enamel surface loss

Biological	**Chemical**	**Health and education**	**Behavioural**
Saliva flow	pH type	Current health	Eating habits
Soft tissue anatomy	Acid type	Socioeconomic status	Drinking habits
Tooth anatomy	Chelation potential	Medication and drugs	Brushing frequency

Source: (Neel *et al.* 2016)

2.2.1 Attrition

Attrition results from tooth-to-tooth contact attributed to occlusal function or parafunction such as bruxism. Studies such as (Trushkowsky, Arias and David 2016) and (Wu, Arsecularatne and Hoffman 2017) reported that enamel attrition can cause loss of tooth structure on the occlusal surfaces and incisal edges. Wu, Arsecularatne and Hoffman (2017) note that attrition occurs between two teeth as well as tooth and enamel fragments generated due to wear. It is also acknowledged in the literature that clenching of teeth can wear out the enamel (Wu, Arsecularatne and Hoffman 2017).

2.2.2 Abrasion

Abrasion is a result of friction between the enamel and any foreign substances which can include toothpaste and or toothbrush (Wu, Arsecularatne and Hoffman 2017). For instance, the abrasives in tooth toothpaste may potentially wear the enamel surface (Onwubu *et al.* 2020a). Another way the enamel could be abraded is by holding hard objects between teeth, for example, holding pens and paper clips, or by chewing rough or hard substances (Field 2012).

2.2.3 Abfraction

Abfraction is often referred to as tooth surface loss that occurs within the cervical portion of teeth. Michael *et al.* (2009) attribute the loss of tooth due to abfraction to excessive bite forces loading tooth structures resulting in cuspal flexure. This may be connected to the high stress that is concentrated in the cervical region, particularly where the enamel layer is thin (Wu, Arsecularatne and Hoffman 2017). According to Field (2012), the flexural force undermines the thinner cervical portions of enamel and this causes the enamel to fracture off the tooth surface.

2.2.4 Erosion

Enamel erosion involves dissolution by acids which are not of bacterial origin (Field 2012). Erosive agents with exogenous acids include carbonated drinks, citrus fruits, alcoholic drinks, yoghurt, and dairy products among others (Jain *et al.* 2012). Erosive agents with endogenous acids enter the mouth through reflux or gastro-oesophageal regurgitation. When the tooth is exposed to an erosive solution, the acid will diffuse through the enamel pellicle to react with the enamel surface (West *et al.* 2017).

Calcium hydroxyapatite will dissolve to form calcium, phosphate, and hydroxyl ions in the solution.

$Ksp = (Ca)_5\,(PO_4)_3\,OH \leftrightarrow 5\,Ca^{2+} + 3PO_4^{3-} + OH$

From a chemistry perspective, H+ ions in the erosive solution are responsible for the demineralization of tooth minerals and will react with ions from calcium hydroxyapatite to form new ions. The equilibrium will change, and the reaction will favour the dissolution of tooth minerals (Onwubu, Mdluli and Singh 2019). When acid encounters the tooth, it not only causes bulk loss of the hard tissue but also softens the remaining surface. Since dentin is softer than enamel, it is more prone to erosion and irreversible loss, exposing dentinal tubules (Onwubu, Mdluli and Singh 2019).

Given the role erosive substances and other abrasive agents play in tooth enamel demineralization and tooth vulnerability, it is critical to understand the process of DH development to aid the researcher in formulating a remedy for its arrest and management. The next section, therefore, explores the process of DH development

2.3 PROCESS OF DH DEVELOPMENT

As reported in the literature (Borges, Barcellos and Gomes 2012), the onset of DH occurred in two phases, namely:

1. **Lesion localization** – The dentin starts presenting hypersensitivity only when exposed to the mouth environment, after the wearing of the protection structures. Enamel loss may result from several factors, while the gingival recession is most often attributed to either overzealous tooth brushing in a healthy mouth or periodontal disease and or periodontal treatment. Enamel loss with subsequent dentin exposure occurs with tooth wear processes like attrition, abrasion, erosion and abfraction (Savage *et al.* 2018). It is worth noticing that not all exposed dentin is sensitive. However, their calcified smear layer, as compared to non-sensitive dentin, is thin and this leads to an increase in fluid movement and consequently, the pain response (Davari, Ataei and Assarzadeh 2013).
2. **Lesion initiation** – in this second phase, the localization should be initiated. If this occurs after the tubular plugs and the smear layer are removed and consequently, dentin and pulp are exposed to the external environment (Miglani, Aggarwal and Ahuja 2010).

2.4 THEORIES OF DENTIN HYPERSENSITIVITY

Based on the performed studies, different theories have been proposed on dentin hypersensitivity namely, direct innervation theory, odontoblast receptor theory and Brännstrom's theory.

1. **Direct Innervation (DI) Theory -** It has been suggested that the nerve endings enter the dentin through the pulp and extend to the dentin-enamel junction (DEJ), where the mechanical stimuli directly transmit the pain (Davari, Ataei and Assarzadeh 2013). However, there is little evidence to prove this theory.

2. **Odontoblast Receptor (OR) Theory -** odontoblasts function as receptors of pain and transmit signals to the pulpal nerves. However, because odontoblasts' cellular matrix is unable to stimulate and generate neural impulses, this theory has also been rejected (Miglani, Aggarwal and Ahuja 2010).

3. **Hydrodynamic Theory** - This theory is the most widely accepted theory for DH and was first proposed by Brännström, Lindén and Åström (1967). According to

> Brännstrom's hydrodynamic theory, when an appropriate stimulus is applied to the outer dentin surface, there is a displacement of the contents of the dentinal tubules, which gives rise to mechanical stimulation of the pain at the pulpodentinal border (Brännström 1986).

In this study, the management of DH is benchmarked on the premise of the hydrodynamic theory. This is because the theory is widely supported by scanning electron microscope (SEM) evidence; which reveals that dentinal tubules in a 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 (Chivu-Garip *et al.* 2012; Davari, Ataei and Assarzadeh 2013; Moura *et al.* 2016). In summary, there are three essential features required for dentin hypersensitivity to occur are (1) the presence of exposed dentin surfaces; (2) open tubule orifices on the exposed dentin surface; and (3) patent tubules leading to a vital pulp. Thus, the ideal management of DH will be the closing of the patent tubules.

2.5 MANAGEMENT OF DENTIN HYPERSENSITIVITY

Special focus must be set on the prevention of DH, such as avoiding erosive drinks or foods and choosing nonabrasive toothbrushes and brushing techniques. Extensive research has been conducted regarding DH treatment, although no single treatment is accepted universally. The management of DH can be divided into two different approaches: self-performed therapy at home (over the counter) or in-office treatment (Davari, Ataei and Assarzadeh 2013). The latter normally apply more sophisticated non-invasive or invasive methods using professional materials and techniques. In general, all interventions should start with non-invasive, reversible, non-hazardous, easy to perform and inexpensive options (Schmidlin and Sahrmann 2013). Only if they prove to be ineffective at revaluation should more invasive interventions be considered.

Although there aren't any established gold standard treatment modalities for the management of DH (Schmidlin and Sahrmann 2013), the use of tubule-blocking agents is gaining popularity as a productive method for the management of DH in the healthcare sector. Home-use desensitizing toothpaste is considered the first choice because of its advantages of wide availability, high cost-effectiveness, and convenient application (Blatz 2012). For immediate alleviation of mild to moderate symptoms, occlusion of dentinal tubules can be noninvasively achieved with toothpaste containing different materials such as 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). Other ingredients used as active ingredients which can plug dentinal tubules directly or after chemical reactions include fluorides (Petersson 2013), calcium phosphate/carbonate (Arnold, Prange and Naumova 2015; Medvecky *et al.* 2018), bioactive glass (Da Cruz *et al.* 2018), strontium acetate (Arnold, Prange and Naumova 2015), or casein-phosphopeptide-amorphous calcium phosphate (CPP-ACP) (Mahesuti *et al.* 2014).

However, the dentin tubules occluded by some of these materials are superficial with limited infiltration depth showing poor acid resistance (Arnold, Prange and Naumova 2015). Equally, these traditional applications are usually challenged by a short life due to daily tooth brushing, saliva dissolving and an acidic diet (Wang *et al.* 2010; Yu *et al.* 2016). Due to the drawbacks, a novel approach to the treatment and management of DH using various combinations of nanoparticles has been advocated in the literature (Tian *et al.* 2014; Wang *et al.* 2014). The rationale behind this strategy, according to Tian *et al.* (2014), is that nanoparticles can easily enter dentin tubules where they may act as mineralizing agents that, when combined with other substances, prevent fluid from moving through the tubules.

2.5.1 Nanomaterials in healthcare

The impact of nanotechnology in society has subsequently led to the development of a variety of synthesis techniques for producing nano-sized materials. Nanomaterials are those materials with components less than 100 nm in at least one dimension, including clusters of atoms, grains less than 100 nm in size, fibres that are less than 100 nm in diameter, films less than 100 nm in thickness, nanoholes, and composites. As revealed in the literature, the unique physical features of the nanoparticles such as the volume/surface ratio yield several advancements in the fields of drug delivery, tissue regeneration, and bioimaging (Donnelly *et al.* 2018; Jain 2020).

Nanotechnology offers a unique approach to overcoming the shortcomings of many conventional materials (Kovtun *et al.* 2012). Among the nanomaterials that have been explored in the literature for the management of DH include mesoporous silica and nanohydroxyapatite. However, these materials are synthesized using lengthy processes and using toxic solvents (Mondal *et al.* 2010). This subsection, therefore, motivates the need to extract nanohydroxyapatite from biowaste and the application of the mechanochemical method in the modification process.

2.5.1.1 Mesoporous Silica Nanoparticles (MSNs).

MSNs have attracted quite a bit of attention over the years. Mesoporous silica has unique properties such as ordered pore structures, high specific surface areas and their synthesis in a wide range of morphologies (Narayan *et al.* 2018), which, unlike traditional porous silica, exhibits exceptionally ordered pores. The unique properties of mesoporous silica nanoparticles including high chemical stability make nanoparticles highly attractive as drug carriers, diagnostic catalysis, separation, and sensing (Ying 2006). More importantly, it has been noted in the literature that the rapid internalization by animal and plant cells without causing any cytotoxicity inside the body, is another distinctive property of surface-functionalized mesoporous silica nanoparticles (Kwon *et al.* 2013). These nanoparticles have superior features compared with organic and inorganic nanostructures such as their tuneable porosity and excellent biocompatibility, and high specific surface area (Wang *et al.* 2015).

MSNs show superior osteoconductivity in comparison to the solid microparticle, and improved bioactivity versus conventional bioglass particles due to a faster release of Si ions (Arcos and Vallet-Regí 2010). In a study by Yu *et al.* (2016), the authors showed that a novel biocomposite based on the medication of nanohydroxyapatite and mesoporous silica nanoparticles (nHAp@MSN) was highly efficient in occluding dentinal tubule with the occlusion showing high acid-resistant stability. They further attributed the acid-resistant stability of the biocomposite to the unique acid resistance of mesoporous silica (Yu *et al.* 2016). MSNs are one of the most versatile and successful particles for biomedical applications. The large surface area, the aspect ratio between pore size and porosity, and the tunability of these characteristics give MSNs advantages over other nanoparticles in the biomedical space (Manavitehrani, Schindeler and Parviz 2018). It was therefore practical to modify nanohydroxyapatite particles with mesoporous silica to enhance the acid resistance characteristics of the biomaterial.

2.5.1.2 Hydroxyapatite (HAp)

Hydroxyapatite [$Ca10(PO_4)_6(OH)_2$] is one of the materials and has gained wide acceptance in medicine and dentistry in recent years (Hannig and Hannig 2010). HAp is the main constituent of dental tissues represented in enamel and dentin (95% - 97% wt.) and 75 % respectively and is responsible for the mechanical behaviour of dental tissues (Khetawat and Lodha 2015). Hydroxyapatite is shown to be a significant material for biomedical applications due to its biodegradability, biocompatibility, and bioactivity (Pokhrel 2018). The nanohydroxyapatite crystals (nHAp) are similar to the apatite crystal of tooth enamel in structure and composition (Huang, Gao and Yu 2009). Thus, nHAp is widely recognized as a bionic material to reconstruct tooth enamel due to its unique capacity for remineralization (Huang *et al.* 2011; Min, Kwon and Kim 2011).

More importantly, nHAp can integrate with tooth or bone structures and support ingrowth without breaking down or dissolving (Wahl and Czernuszka 2006). It acts not only by obliterating the dentin tubules and by precipitating on the surface and inside the dentin tubules but also by the depolarization of nerve endings. Pokhrel (2018) suggested that nanoparticles are useful for applications such as fillers for composites, reparative materials for damaged enamel and carriers for drugs. In line with this recommendation, a few recent studies have shown that this calcium phosphate is used in oral care formulations and offers several benefits, such as effective reduction of teeth sensitivity (Browning, Cho and Deschepper 2012), fast enamel remineralization (Gjorgievska *et al.* 2013; Nasution and Gani 2017), improved smoothness of tooth surface and whitening (Takikawa *et al.* 2006; Jiang *et al.* 2008).

It is worth noting that the HAp nanoparticles (nHAp) are more useful than conventional-sized HAp bulk ceramics based on the higher surface-to-volume ratio, chemical reactivity, and biomimetic morphology. Unlike micro-sized HAp, the tiny nHAp is easily integrated into the dentinal tubules, enhancing their occlusion (Priyadarsini, Mukherjee and Mishra 2018). The nHAp seals the tubules and prevents exposure of the nerves to external stimuli, thereby reducing dental hypersensitivity (Nanoxim White paper 2013). This is evident in the new home-use desensitizing products containing nHAp that have been formulated and adopted by more patients. When observing under a Scanning Electron Microscope (SEM), nHAp-containing toothpaste showed great sealing ability (Kulal *et al.* 2016). Furthermore, clinical studies proved that nHAp-containing toothpaste could reduce DH significantly (Suresh *et al.* 2018; Vano *et al.* 2018), thus supporting its recommendation in patients' daily use (Pei *et al.* 2019).

2.5.1.3 Motivation for nanohydroxyapatite-silica composite and the use of biowaste

A recent study by Yu *et al.* (2016) claimed that the mesoporous silica and hydroxyapatite composite offers better dentin tubule occlusion with outstanding resistance to acidic attack. However, the method of synthesizing the MSN@nHAp composite is noted to be cumbersome, time-consuming, and requires the use of toxic chemicals. Hence this study looks at an environmentally friendly and cost-effective method of synthesizing MSN@nHAp using mechanochemistry. Abdulrahman *et al.* (2014) reasoned that using environmentally friendly materials to treat DH, would strengthen the economic benefits associated with using natural waste material, which is high on the global agenda for a greener environment. Yazıcıoğlu and Ulukapı (2014) advised that low-cost, affordable, possible, and sustainable products should be developed to repair and improve the quality of life for patients suffering from a dental problem such as tooth sensitivity. It is mostly recognized that instant relief is a highly motivating factor for DH patients and that complete and robust dentin occlusion is the most promising treatment strategy for achieving relief. Chivu-Garip *et al.* (2012) suggested that desensitizing agents need to be non-irritant to the pulp, painless on the application, easily applied, rapid action, effective for an extended period, without staining effects and consistently effective. In line with their advice, this study looks to synthesize nanohydroxyapatite from natural resources.

2.5.2 Relevant literature on hydroxyapatite used in a toothpaste formulation

Due to their distinctive properties, nHAp has grown in popularity among researchers over time. A summary of studies on hydroxyapatite, including their chemical properties and their effectiveness in treating dentin hypersensitivity under different conditions, is presented in Table 2.2.

Table 2. 2: Research on nHAp applications in toothpaste formulations

Author	Type of Study	Source of nHAp	Key points
Amaechi *et al.* (2015)	Double-blind – In situ	Commercial product	Dentifrices containing nanohydroxyapatite occluded dentin tubules, demonstrate the potential to relieve dentin hypersensitivity.
Bologa *et al.* (2020)	In vitro	Commercial product	In their study, SEM analysis of dentin samples treated with all three n-HAp kinds of toothpaste revealed mineral deposition that covered the intertubular dentin and blocked the dentinal tubules.
Amaechi *et al.* (2021)	In vivo	Commercial product	In comparison to sodium fluoride at 1450 ppm, toothpaste containing 10% HAp was marginally more effective in preventing tooth demineralization. Their research demonstrated that HAp toothpaste can effectively replace fluoride toothpaste for the prevention and treatment of dental caries.
Onwubu *et al.* (2020b)	In vitro	Eggshells	The results of their study indicate that nHAp can be successfully produced using an efficient environmental technique. It is also conceivable that the nHAp produced will be a highly effective component of toothpaste formulations for both the potential treatment of tooth sensitivity and enamel remineralization.

Low, Allen and Kontogiorgos (2015)	Clinical	Commercial Product	According to the outcomes of their clinical study, using a toothpaste with potassium nitrate, sodium monofluorophosphate, nano-hydroxyapatite, and the anti-inflammatory agents phloretin, ferulic acid, and silymarin daily can significantly and quickly minimize tooth pain caused by dentin hypersensitivity.
Kulal *et al.* (2016)	In vitro	Commercial product	Their research revealed that nano-hydroxyapatite had a 98.1% tubule occlusion efficacy. Additionally, nano-hydroxyapatite toothpaste performed more effectively than other desensitizing agents in their study.
Roveri *et al.* (2009)	In vitro	Synthetic	Biomimetic hydroxyapatite nanocrystals were found to be capable of remineralizing the surfaces of dentine that were etched by the application of orthophosphoric acid and of gradually occluding dentine tubules in a few minutes until the regeneration of a layer of mineralized tissue in a matter of hours.
Shetty, Kohad and Yeltiwar (2010)	Clinical + in vitro	Synthetic (sol-gel + precipitation) + commercial product	Although all treatment groups saw a significant reduction in hypersensitivity symptoms, HAp showed a definite potential as an effective desensitizing agent that provides immediate symptom relief.
Ali, Al Saffi, and NIAMA DAYEM (2013)	In vitro	-	Their research demonstrated that, when compared to other treatment modalities, the use of nano-fluor-hydroxyapatite (NFH) and an Nd: YAG laser was the most effective method of sealing off exposed dentinal tubules and could be a promising modality for managing dentin hypersensitivity.
Tschoppe *et al.* (2011)	In vitro		In their research, toothpaste with n-HAp demonstrated greater remineralizing effects in comparison to toothpaste with amine fluoride using bovine dentine, and similar trends were observed for enamel.
Browning, Cho and Deschepper (2012)	Clinical trial	Commercial product	In their study, they found a statistically significant decrease in the number of days of tooth sensitivity experienced during active bleaching when using the n-HAp toothpaste.
Vano *et al.* (2014)	Double-blinded - Clinical trial	Commercial product	Comparable to or even superior to fluoride toothpaste, nHAp toothpaste demonstrated remineralizing effects. As a result, the use of nHAp has been encouraged as an efficient desensitizing agent that provides rapid relief.
Al-maliky *et al.* (2014)	In vitro	Commercial product	Their research demonstrated that the occlusion of dentinal tubules and reduction in the permeability of exposed dentin occurred when a moderate power density CO_2 laser was used in conjunction with nanohydroxyapatite paste.
Vano *et al.* (2018)	Double blinded-clinical trial	Commercial product	They concluded that nano-hydroxyapatite is an efficient desensitizing agent that relieves symptoms after two and four weeks when used in fluoride-free gel toothpaste.

Baglar *et al.* (2018)	In vitro	Synthetic (precipitation)	The evaluated nHAp types offered a strong tubular plug occluding layer on the dentine surface. The resistance of this layer to degradation was also confirmed.
Narmatha and Thakur (2014)	In vivo	Commercial product	They concluded that propolis and nanohydroxyapatite could be used as a treatment for dentin hypersensitivity. Propolis and nano-hydroxyapatite both are effective natural alternatives for treating dentin hypersensitivity.
Wang *et al.* (2016)	Double blinded-clinical trial	Commercial product	They discovered that Nano-hydroxyapatite formulations (with or without home-care product association) reduced dentin hypersensitivity just as effectively as the other treatments during the test period.
Shetty and Kundabala (2013)	Double blinded-clinical trial	Synthetic (sol-gel + precipitation)	When employed as an in-office procedure, HAp demonstrated potential as an efficient permanent desensitizer when compared to potassium nitrate and sodium monofluorophosphate.
Huang *et al.* (2011)	In vitro	Synthetic	They concluded that nano-HAp has greater remineralization effects than micro-HAp under neutral conditions and has good potential for remineralizing initial enamel caries lesions under dynamic pH-cycling conditions. Additionally, they discovered that, when used in acidic environments, nano-HAp can significantly speed up the rate, depth of penetration, and extent of remineralization of artificial incipient lesions.
Suresh *et al.* (2018)	Double blinded-clinical trial	Commercial product	The study promoted the use of dentifrices containing arginine and nano-hydroxyapatite as an efficient desensitizing agent for treating DH symptoms. Additionally, the nHAp group consistently displayed a greater reduction in DH at each of the time frames.
Gopinath *et al.* (2015)	Double blinded-clinical trial	Commercial product	In comparison to previously tested benchmark toothpaste, their study revealed that HAp-containing toothpaste was effective in reducing dentin hypersensitivity, and as a result, it can be recommended for the management of hypersensitivity.
Jena and Shashirekha (2015)	Double blinded-clinical trial	Commercial product	After a single application for up to four weeks, toothpaste containing 15% n-HAp was found to be the most successful at reducing DH, followed by those containing 8% arginine and 5% NovaMin.
Douglas de Oliveira, et al., 2016	Double blinded-clinical trial	Commercial product	They discovered that only toothpaste containing calcium phosphate nanoparticles in the form of hydroxyapatite provided an immediate relief effect.

It is apparent from the findings of the studies mentioned above that nHAp is a useful material that can be used for DH management. Most other studies used commercial products whose source of nHAp is synthetic; only the study by Onwubu et al. (2020) mentioned the use of natural materials to produce the hydroxyapatite. Furthermore, studies compared the ability of nHAp to remineralize or occlude open dentin tubules to toothpaste that contained fluoride rather than one made from a natural resource. Silica is well known for its characteristics in an acidic environment. Therefore, the study used silica to produce a nHAp@MSN composite to ensure that the nHAp's efficacy is not affected by low pH levels in the mouth. This study looks to not only produce nHAp from various natural waste materials but also to compare the properties and effectiveness of the nHAp in managing DH due to the growing concern over global warming and the opportunity to turn waste into a value-added product. Utilizing natural resources not only ensures that the product is safe to use but also that it can be made affordable, as well as benefit the ecosystem.

2.6 SOURCES OF NANOHYDROXYAPATITE

This study proposition was to extract nHAp from biowaste eggshells and fish scales. Part of the inquiry the research aim to address was to assess and compare the physicochemical properties and characteristics of the nHAp extracted from eggshells and fish scales. This is vital as the researcher aims to establish a benchmark characteristic in the nHAp extracted from biowaste for medical and dental applications. Natural hydroxyapatite is usually extracted from biological sources or wastes such as mammalian bone, marine or aquatic sources, shell sources, plants, and algae and from mineral sources as shown in Table 2.3. Nevertheless, the researcher aims to extract hydroxyapatite only from fish scales and eggshells. This is motivated by the abundant availability and can be collected in plenty in Durban, South Africa. Thus, this section reviews related literature on a natural source of nHAp with emphasizes on eggshells and fish scales.

Table 2. 3: Common natural source of hydroxyapatite

Mammalian	Aquatic	Shells	Plant and algae	Mineral
Bone a. Bovine b. Horse c. Camel d. Pig	Fish bone Fish scales	Cockle shell Seashell Clam shell Mussel shell	Algae Peel Fruit Woods Plant (stalk, leaves, and flowers	Limestone

Source (Pu'ad *et al.* 2019)

2.6.1 Eggshells

Eggshells are an inexpensive and abundant material containing calcium carbonate in the form of calcite, which is the main component of bones and teeth (Cree and Rutter 2015; Onwubu 2016). Tons of eggshell waste is produced every day and most of this waste is not effectively managed leading to negative impacts on the environment (Abdulrahman *et al.* 2014). Bitzer and Sims (1988) reported that eggshells used for cropping cause nitrate contamination of groundwater. This is concerning as there is a strong correlation between the level of nitrate in drinking water and the occurrence of blue baby syndrome, cancer, respiratory illnesses in humans and foetal abortions in livestock. Given the amount of eggshell waste that needs to be disposed of worldwide, it is important to find alternative means of converting eggshell waste materials into value-added products for environmental sustainability (Faridi and Arabhosseini 2018).

Of significance and vital to this study, trace amounts of ions such as Na^+, Sr^{2+} and Mg^{2+} found in eggshells make them a useful waste material for the synthesis of Hydroxyapatite (HA). As previously mentioned, Hydroxyapatite is a significant material for biomedical applications due to its biodegradability, biocompatibility, and bioactivity. In their study, Nuamsrinuan *et al.* (2017) concluded that nanohydroxyapatite could be synthesized using calcium that originated from chicken eggshells by ball milling technique. In their study nanohydroxyapatite appeared after 5 min ball milling time but the product was of low crystallinity and crystalline size, to synthesize the optimal nanohydroxyapatite from waste eggshell, the results indicated a ball milling time of about 50 to 60 min. The process of transforming eggshells into hydroxyapatite and nanohydroxyapatite utilizing mechanochemistry is environmentally friendly. Abdulrahman *et al.* (2014) reported that eggshell-based hydroxyapatite and nanohydroxyapatite stand a good chance of reducing the cost of treatment of DH with a minor impact on the environment.

The use of waste eggshells has been dominant among the alternative procedures. Indeed, producing HAp from waste eggshells has advantages: (1) it reduces the cost of production; (2) it offers HAp that is very compatible with the human bone and hard tissues; and (3) it is an environmental and waste control measure. Traditionally, the eggshells are dumped or incinerated after consumption of the egg contents. This is a big environmental issue; however, it is also an immense biological calcium (bio-calcium) source for countless applications. One such application (as stated) is a conversion of waste eggshells into HAp. Nonetheless, previous procedures for converting eggshells into HAp have generated impure products with larger particle sizes, low surface areas and extremely low pore volumes (Ibrahim *et al*. 2015). This study will be looking at improving the method of extracting pure nanohydroxyapatite from eggshell waste.

2.6.2 Fish Scales

The rise in seafood consumption has given rise to an increase in waste generated from seafood processing plants. Most of this waste is either improperly discarded or land filled which ultimately pollutes the environment (Singh *et al.* 2022). However, this waste which mainly includes the head, bones, viscera, and scales is abundant in proteins, lipids, and other bioactive compounds. Venugopal (2021) notes that these solid wastes have tremendous potential to produce value-added products and chemicals such as chitin, pigments, proteins, fatty acids, glycerol, and alcohol, among others. Of interest, HAp can be derived from natural materials such as fishbone (Ozawa and Kanahara 2005) and fish scale (Mondal *et al.* 2010; Zainol *et al.* 2012).

The main by-product of the seafood processing industry is fish scales with a portion of 30-40 % of the total amount and managing those by-products is causing problems for the companies (Gumisiriza *et al.* 2009). The conversion of fish scales has dual benefits: extraction of HAp and other useful products as well as solid waste management of the fishery industry. Besides, fish sources are much safer, and the wide evolutionary gap between fish and humans suggests a minimal risk of disease transmission (Venkatesan *et al.* 2015). This study, therefore, aims to utilize the fish scales to produce hydroxyapatite through mechanochemistry.

2.7 METHODS OF HYDROXYAPATITE EXTRACTION

There are several methods of preparing HAp crystals reported in the literature, these methods can be classified into three groups: dry methods, wet methods, and high-temperature processes. While this study acknowledges the several methods of extracting HAp from biowastes such as eggshells and fish scales (Table 2.3), the focus of this study will be the use of the mechanochemical method in the preparation and extraction of valuable biobased materials from waste. This section, therefore, details the mechanochemical process used in this study.

Table 2. 4: **Hydroxyapatite extraction methods**

Methods		
1. Dry methods	2. Wet methods	3. High-temperature processes
a. Solid state b. Mechanochemical	a. Chemical precipitation b. Hydrolysis c. Sol-gel d. Hydrothermal e. Emulsion f. Sonochemical	a. Calcination b. Pyrolysis

(Researcher's creation)

2.7.1 Mechanochemistry

The need for cleaner, safer, and more sustainable chemical transformations is the original impetus for the current expansion of solid-state methodology, especially as raw materials become increasingly scarce. To this end, a simple strategy is to eliminate or reduce solvent usage throughout any designated synthetic routes (Friščić, Mottillo and Titi 2020). Mechanochemistry is the study of chemical and physicochemical transformations of substances in all states of aggregation caused by the action of mechanical energy (Hua *et al.* 2017). Mechanochemistry has a number of advantages over traditional methods such as calcination, hydrothermal reactions, and solution-based chemistry (Ojeda *et al.* 2014). Thus, the use of this method has attracted the attention of scientists working in nanomaterial fabrication and organic synthesis (Stolle *et al.* 2011; Schreyer *et al.* 2019; Onwubu *et al.* 2020b).

Baláž (2018) notes that the application of the mechanochemical method in composite material preparation has gained widespread interest among researchers, due to its simplicity, environmental friendliness, and versatility. Hua *et al.* (2017) revealed that the mechanochemical method utilizes mechanical force to effect chemical reactions or structural changes of material like those observed for thermochemistry (energy by heat), photochemistry (energy by light), and electrochemistry (energy by electrical potential. Performing reactions without the need for solvents is also a huge benefit, as it reduces the cost of a process by eliminating a waste source. The benefits over solvent-based methods also include shorter reaction times, lower temperatures, less workup required, and the possibility to perform one-pot syntheses, all while obtaining equivalent or higher yields (Amrute, Zibrowius and Schüth 2020).

As documented in the literature, the application of mechanochemistry has the advantage to cause both structural, and particle size reductions in material preparation (Wu and Li 2012). Accordingly, the mechanochemical method is assumed to be a powerful tool for the modulation of chemical activity as well as the preparation of materials with high performance (Wiggins, Brantley and Bielawski 2013). Despite the enormous potential of the mechanochemical method, there is, however, limited evidence of its use for the preparation of mesoporous silica and nanohydroxyapatite composite (MSN@nHAp). This study, therefore, intended to use a mechanochemical method through the application of a ball-milling process to synthesize a mesoporous silica-nanohydroxyapatite bio nanocomposite (MSN@nHAp) from natural resources for its potential application in the management of DH.

2.7.2 Optimizing the mechanochemical method

Of interest, when performing any reaction, understanding and controlling the variables are important (Liptak 2018). However, when using a ball mill it can be challenging to control or even measure the variables individually (Howard, Cao and Browne 2018). Three main variables affect how mechanochemical reactions perform: the kinetic energy of the ball(s) prior to the collision, how that energy is transferred to the reagents and the frequency of collisions (Leonardi, Villacampa and Menéndez 2018). The amount of kinetic energy that the ball(s) possess prior to the collision is the maximum amount of energy that can be transferred to the reagents per collision. In a collision, how that energy is transferred can have an effect on whether or how are action occurs (Cagnetta *et al.* 2016).

This can be by a direct impact, under which the material is locally compressed, or by shear force in which a reactive face is exposed (Cheng, Hernández and Bolm 2018). It has been shown that these different types of energy absorption can lead to different outcomes (McMahon *et al.* 2011). Different types of ball mills achieve different ratios of impact and shear forces (Andersen and Mack 2018). Differences in mixing or mass transfer can affect the outcome of any reaction (Makinde and Animasaun 2016). In solution, this is easily controlled by stirring, mass transfer is rarely a problem on a small scale (Chiu *et al.* 2017). However, in milled reactions, this can be a difficult variable to control and may have a dramatic effect on the outcome of a reaction (Achar, Bose and Mal 2017). To help homogeneity and mixing, grinding agents can be used (Kulla *et al.* 2018). Finally, those variables that are usually changed in any standard reaction optimisation also apply, such as stoichiometry, reaction time and temperature (Hutchings *et al.* 2017).

Moreover, the temperature is not a parameter that is easily controlled, the reaction vessel heats up due to the collisions and has a dependence on the milling degree volumes of grinding balls, sample and vessel size as well as oscillating frequency (Loh, Samanta and Heng 2015). This has a significant effect on the trajectories of the balls, and therefore the energy transfer and mixing (Ferguson *et al.* 2019). The effect of the milling ball milling degree has been investigated for the Knoevenagel condensation of vanillin with barbituric acid in a planetary mill (Schmidt *et al.* 2015). For this specific example it was observed that having the balls milling approximately 25% of the total volume was optimal (House *et al.* 2015). However, this may not be the case for other reactions and/or other types of mills (Colacino *et al.* 2018). The milling frequency is perhaps the easiest variable to control and can be changed simply by adjusting the settings on the mill (Jug and Mura 2018). When increased, this increases the velocities of the balls, and so increases their kinetic energy (Ghayour, Abdellahi and Bahmanpour 2016). This is a facile way to change the energy input into a reaction (Muñoz-Batista *et al.* 2018). Cognisance of the temperature, and the number of milling balls, will be taken by the researcher; during the nHAp preparation using the mechanochemical method.

2.8 CONCLUSION

The chapter presents relevant literature related to the management of DH and motivates the use of biowaste as a sustainable material for the extraction of value-added products. The review begins with an overview of the structure of the human tooth. It was highlighted that the human enamel which serves as protection for the tooth is susceptible to damage by both external and internal factors. The consequence of this contributes to the onset of DH and thus requires effective management. The review argued and motivated the use of nanomaterials, particularly nanohydroxyapatite in the management of DH. The rationale behind the use of biowaste for the extraction of nHAp was provided and explained. The foregoing reviewed literature further highlighted an eco-friendly strategy for the preparation of nHAp and its modification with silica through the mechanochemical method. The next chapter will provide the underlining theoretical framework adopted to evaluate the product quality of nHAp extracted from biowaste.

CHAPTER THREE – RESEARCH METHODOLOGY AND DESIGN

3. 1 INTRODUCTION

This chapter presents the research design and approach adopted in the study. The research design included multiple phases, the first of which looked at a clear strategy to develop bio nanocomposite using nano-sized hydroxyapatite and mesoporous silica. Subsequently, the methodology used in phases two and three, that is the various experimental work and analyses that were conducted were described in-depth.

3.1.1 Background to the research methodology

Research is generally stimulated by a number of methods such as a scientific method of inquiry and critical thinking, deductive reasoning, and inductive reasoning amongst others (Welman, Kruger and Mitchell 2005; Berg and Latin 2008). Deductive reasoning provided the basis for the initiation of this study. Rahardjo *et al.* (2015) argued that using occluding products that physically block the exposed dentin tubules is highly effective in demineralizing enamel lesions, particularly in toothpaste. In line with the suggestion in the literature that an inexpensive, non-hazardous, and non-invasive material should be developed for the management of DH (Schmidlin and Sahrmann 2013), this study sought to comparatively assess and evaluate the dentin remineralisation characteristics of nHAp extracted from fish scales and eggshell wastes.

This study, therefore, follows a quantitative research approach and an experimental research design strategy. alleged that a quantitative approach tests “hypotheses with empirical data to see if they are supported” (Johnson and Christensen 2019). 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 2019). To facilitate the proposed research design and methodology, a three-phase research methodology was considered most appropriate (Figure 4-1).

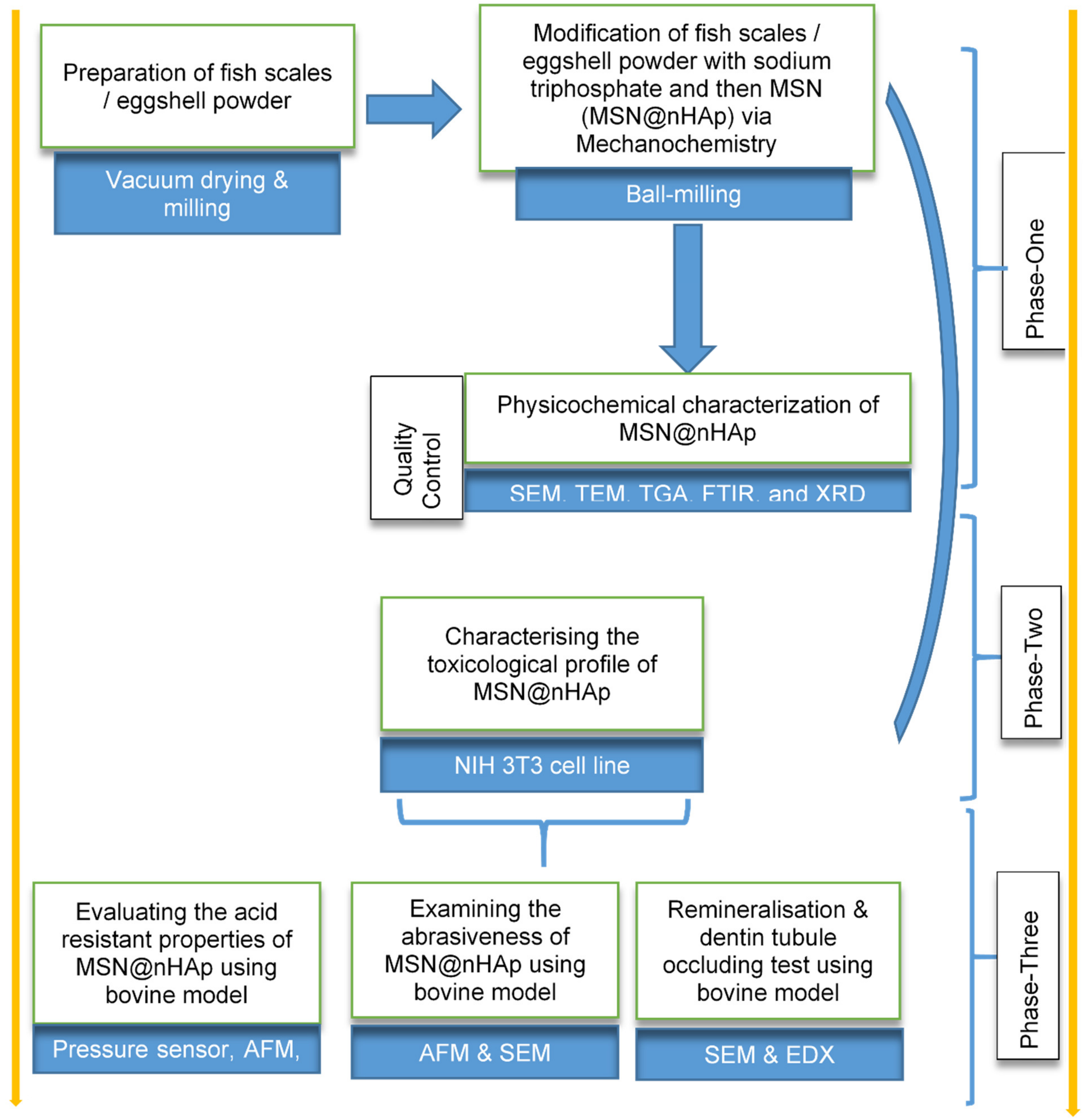


Figure 3. 1**:** Experimental research design outline

3.2 MATERIALS AND METHOD

This section details the materials and various methods followed in the extraction of nHAp from eggshells and fish scales and the subsequent modification of the nHAp extracted with silica to obtain MSN@nHAp.

3.2.1 Phase One: Preparation and extraction of nHAp from biowaste materials

Nanohydroxyapatite from eggshells and fish scale waste were extracted and characterised as described in the sections below

3.2.1.1 Preparation and extraction of nanohydroxyapatite from eggshells (EnHAp)

In preparation for EnHAp from waste eggshells, 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). In accordance with the techniques demonstrated by Onwubu (2016) the eggshell powder was prepared based on the formulae below:

- **Washing and disinfecting of the eggshells:** The collected eggshells were disinfected in a solution of sodium hypochlorite for six hours for the removal of bacteria such as Salmonella and E-coli.
- **Vacuum drying:** To burn out the membranes, the eggshells were vacuum dried for ± 6-9 minutes at 250 ºC.
- **Ball Milling:** The vacuum-dried eggshells were subsequently crushed into powder (Figure 3.2). This was achieved by placing 30g of the eggshell in a 250ml 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 450 rpm for 1hr minute (Figure 3.2)

Figure 3. 2: Eggshells + 50 stainless steel balls for 1hr = eggshell powder **(cream white)**.

- Mechanical sieving; As shown in Figure 3.3, the milled eggshells were sieved using a mechanical shaker for 60min at a speed of 100 rpm using a sieve with a mesh size of 25 µm.

Eggshell powder

100 amplitude for 60min

Powder sample of <25 µm

Figure 3. 3**:** Mechanical sieving process: (A) powder in a sieving mesh; (B) milling condition; and (C) sieved eggshell powder

- **Calcination of eggshell powder**: Eggshell powder was calcined by heating the prepared eggshell powder in a furnace at 900 ℃ for 3 hours at a heating rate of 3°C/min to obtain a snow-white powder. The snow-white powder obtained was a result of the decomposition of the calcium carbonate constituent of the eggshell waste to calcium oxide following the equation below.

$$CaCO_3 \longrightarrow CaO + CO_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{eq1}$$

$$CaO + H_2O\ (\text{moisture}) \longrightarrow Ca(OH)_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{eq2}$$

- **Extraction of EnHAp:** Using the stoichiometric ratio of 1:5, 20g of the snow-white powder and 13.4g of sodium triphosphate were wetly milled together in a 250mL bowl containing 100mL deionized water at 500 rpm for 5 hours. The mixture was centrifuged at speed of 1000 rpm for 30min after milling (Figure 3.4), which was then oven dried for 5 days at 40 ℃. Milling was done using a planetary ball mill (Retsch® PM 100) with thirty stainless steel balls of 10mm (diameter).

Wet-milled nHAp in a centrifuge

nHAp after 30min

Dried nHAp sample

Figure 3. 4: extracted EnHAp during and after centrifugation

3.2.1.2 Preparation of nanohydroxyapatite and Silica Composite (MSN@nHAp)

Using the planetary ball mill mentioned above, mesoporous silica (0.9 µm; Sigma-Aldrich) was used to modify the extracted EnHAp at 500 rpm for 40 min in a ratio of 1: 10 (1 g of MSN to 10 g of nHAp). The parameters used for the milling process in the two steps include 30 stainless steel balls of 10 mm diameter in a 250 mL bow. The powder obtained after milling was characterised to establish the formation of nHAp in the first step and the subsequent modification with silica in the second step.

3.2.1.3 Preparation and extraction of nanohydroxyapatite from fish scale waste (FnHAp)

Fish scales were obtained in a fish market in Durban central. They were then transferred to a research laboratory where they were washed thoroughly in distilled water and sodium hypochlorite to remove the organic substances. Thereafter, scales were left to air dry in the laboratory conditions until dryness (Figure 3.4).

Fish scales from the market

Washing of fish scales

Drying of fish scales

Figure 3. 5: Fish scale preparation process

The extraction of FnHAp from fish scales was achieved in two batches. In the first batch, the scales were then immersed in 1 M hydrochloric acid (HCl) for 24 hours. After 24 hours. Thereafter, the scales were thoroughly rinsed with reverse osmosis water and subsequently soaked in 1 M sodium hydroxide (NaOH) for another 24 hours to remove proteins. The fish scales were rinsed, placed in a beaker with water, and boiled for 20 minutes on a hot plate at 80 ºC. The fish scales were then dried in an oven at 80 ºC for 2 hours (Figure 3.6).

Acid and base treatment

Heating at 80 ºC for 20 min

Oven drying of fish scales

Figure 3. 6: Alkaline hydrolysis process

The scales were placed in crucibles and heated in a furnace at 700 ℃ for 2 hours (Figure 3.7). In the second batch, the obtained power (FnHAp). Thereafter, FnHAp was dry ball-milled at 500 rpm for 2 hours using the same milling parameters explain above to obtain (mFnHAp).

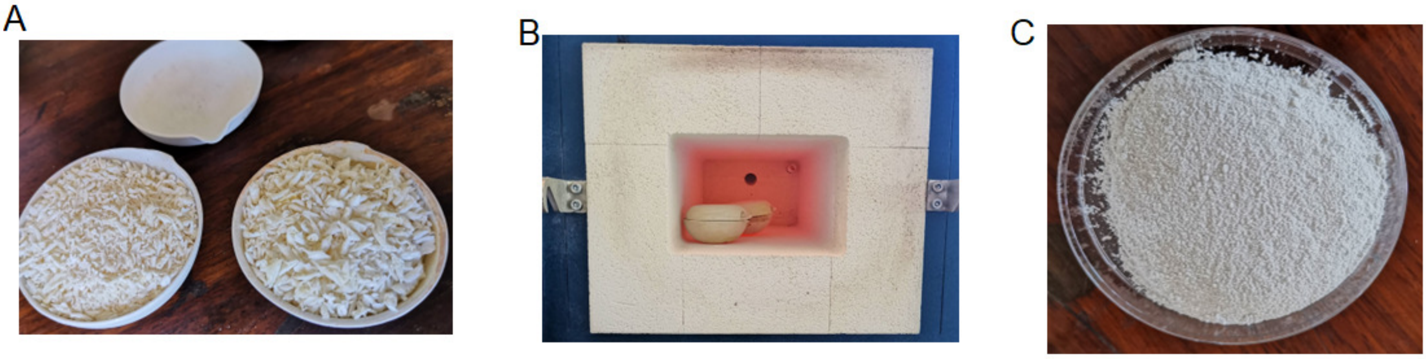


Figure 3. 7: Showing (A) oven dried fish scales (B) calcination (C) calcined FnHAp

3.2.2 Phase Two: Physicochemical characterisation of the extracted nHAp and MSN@nHAp

This phase details various characterisation techniques applied to establish the formation of nHAp from both eggshells and fish scales and the subsequent modification of eggshells nHAp with silica. In compliance with the international standards for quality management and quality assurance (ISO 9001: 2008), the particle size, shape, phase change, and crystallinity of nHAp (EnHAp, FnHAp, and mFnHAp) and MSN@nHAp were determined by using the various analytical techniques. These are detailed in the sections below.

3.2.2.1. Fourier transform infrared spectroscopy analysis

The functional group present in the prepared powders (EnHAp, FnHAp, mFnHAp, and MSN@nHAp) were identified using a Perkin Elmer Universal ATR). Before scanning, an initial background check was performed. Thereafter, a small amount of the prepared sample powders was placed in the sample holder and scanned in the range of 400-4500 cm^{-1} at a resolution of 4 cm^{-1}.

3.2.2.2. X-ray diffraction analysis

The X-ray diffraction (XRD) analysis was performed to observe the possible changes in the crystallinity of the sample powders (EnHAp, FnHAp, mFnHAp, and MSN@nHAp). The XRD patterns were recorded using a diffractometer (D8 Advance BRUKER AXS instrument Germany instrument; Cu-Ka radiation (lKa1=1.5406Å) and analysed between 0 and 90 (2 theta). The voltage, current and pass time used were 40 Kv, 40 mA, and 0.5s, respectively.

3.2.2.3. High-Resolution Transmission electron microscopic analysis

The particle size, shape, and distribution of the prepared powders (EnHAp, FnHAp, mFnHAp, and MSN@nHAp) were studied using a high-resolution transmission electron microscope (HRTEM; Philips CM 120 model) operating at 120kV. Before the HRTEM observation, small quantities of the nHAp were dispersed in 10mL ethanol and sonicated for 10min at 10Kv. Thereafter, thin cross-sections of cryo-microtomed specimens were prepared using a Leica microtome (South Africa) and placed on carbon copper grids of 100mm by 100mm.

3.2.2.4 Elemental analysis of samples

Elemental characterization of the samples on the unexposed and exposed surfaces to the candies was determined using Energy Dispersive X-ray (EDX) Analysis. Four different sites were measured per sample and the mean difference value between the unexposed and exposed surfaces was used for statistical analysis. Results were obtained as a percentage weight of all elements detected. Data for Carbon (C), Oxygen (O), Phosphorus (P), and Calcium (Ca) are presented due to our interest in the elemental change of calcium and phosphorous that forms the enamel mineral crystallites

3.2.2.5. Buffering and pH test

For each of the samples (EnHAp, FnHAp, mFnHAp, and MSN@nHAp), 1.5 g were placed in a beaker containing 50 mL of deionized water. The solution was constantly agitated at a low speed of 600 r/min and the pH was measured at 1-min intervals for a duration of 10 min. A pH meter (BOECO, BT-675, Germany) equipped with a temperature sensor was used to record changes in the pH reading (Figure 3.8). Similarly, the samples were exposed to 2 mol L^{-1} hydrochloric acid (HCl) and the pH was recorded after 10 min.

Figure 3. 8: A typical pH and buffering test set-up

3.3 PHASE TWO: TOXICOLOGICAL ASSESSMENT OF nHAp

This section details the cytotoxicity assessment of the extracted nHAp. This was done as part of the quality and risks assessment process in the use of nanosized materials in toothpaste formulation. As a reference point, only EnHAp was assessed for its biocompatibility with soft tissues.

Using the MTS endpoint assay (3-(4,5-dimethylthiazol2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)- 2H-tetrazolium) the cytotoxicity of EnHAp was assessed in vitro against NIH 3T3 (mouse fibroblast). The cells were incubated and tested in accordance with the procedure described by Onwubu *et al.* (2020b). In this procedure, the NIH 3T3 cells were left to incubate for 4 days, whereupon MTS (5µL) was added to the cells. The absorbance values were measured at 490 nm after 1, 2, and 4h incubation periods, averaged and the viability curves are drawn up. The cells (1×105cells/mL) were incubated in 96 well plates at 37°C overnight, with the subsequent addition of the supplied compounds, in concentrations of (100, 50.0, 25.0, 12.5, 6.25, 3.125, and 0µg/mL. Auranofin was used as a negative control. The samples were tested across three plates in duplicate (n=6) and the average value was reported.

3.4 PHASE THREE: ASSESSING THE “FITNESS OF PURPOSE” OF THE nHAp AND MSN@nHAp

This phase assesses the fitness of the purpose of the prepared nHAp (EnHAp, FnHAp, and mFnHAp) and MSN@nHAp. The phase examines the acid resistance properties of the sample powders against erosive challenge, and the dentin tubule occlusion characteristics.

3.4.1 Acid resistance and remineralisation assessment

The acid resistance and remineralization properties of the powders (EnHAp and MSN@nHAp) were studied using bovine tooth enamel. It was assumed that if powders have improved acid-resistant properties, then it would be a suitable material for the remineralisation and repair of damaged teeth. The tooth enamel was placed in a 4% citric acid solution containing each of the respective powders for 2min and thereafter rinsed with deionized water and blot-dried. The images of the specimens before and after acidic exposure were studied using a scanning electron microscope (Field Emission-Carl Zeiss). Energy-dispersive spectroscopy was further used to quantify the elemental loss in the samples after acidic exposure. Each tooth sample was assessed at four separate locations on the tooth surface, and the mean elemental loss was calculated as a percentage of the total weight of all elements found after acidic exposure.

3.4.2 Assessing the dentin tubule occlusion characteristics of nHAp and MSN@nHAp

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

3.4.2.1 Bovine tooth preparation and sample groups

Forty freshly extracted bovine-enamel anterior teeth were obtained from a slaughterhouse, in South Africa. The collected teeth were subsequently cleaned and disinfected in a 10% chloroxylenol solution. Dentin discs 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. Subsequently, the prepared dentin disc was wetly grounded with silicon carbide polishing papers (600–1000 grits) for 60 s (Figure 3.9). Before simulating the sensitive model, the discs were mounted in a resin (AMT composite, South Africa). Thereafter, dentin tubules were opened by soaking the specimens in 8 wt % citric acid solution for 5 min. As shown in Table 3.1, the specimens were randomly assigned into four groups (n = 10) and treated using agitation and conventional brushing techniques.

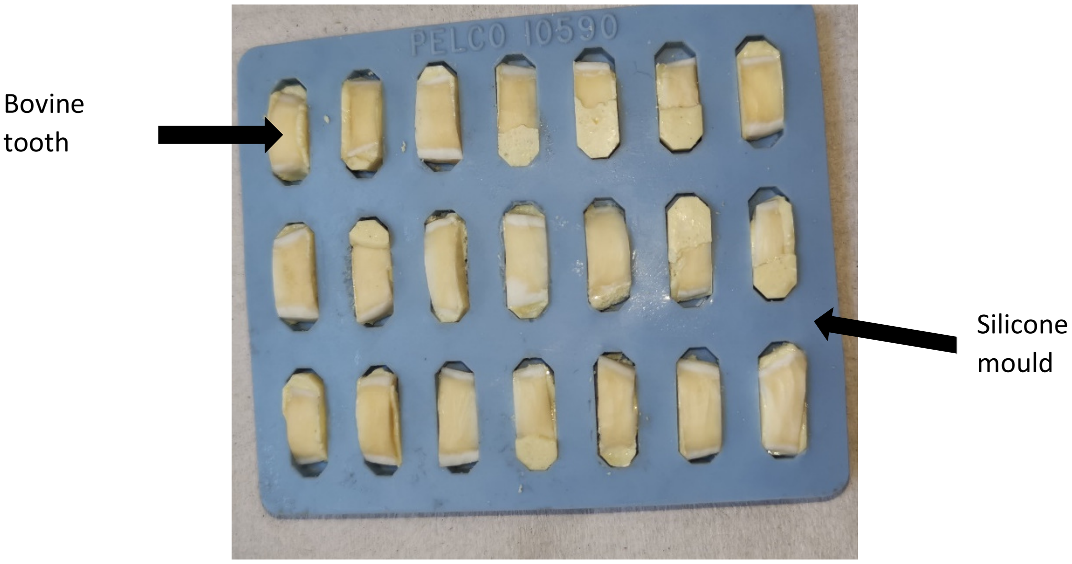


Figure 3. 9: Showing bovine embedded in silicone mould

Table 4.1: Sample groups

Sample powder	Treatment type		Total
	Agitation (n)	Brushing (n)	
EnHAp	5	5	10
FnHAp	5	5	10
mFnHAp	5	5	10
MSN@nHAp	5	5	10

n=Number of bovine teeth

3.4.2.2 Agitation test

The specimens were then agitated in a beaker containing 1 g of the samples (Table 4.1) and 40 mL of deionized water for 5 min. After exposure, the specimens were rinsed with deionized water and blot tried. The specimens were agitated following the protocol described by Onwubu *et al.* (2018). The image of the specimens before and after treatment and exposure were studied using SEM.

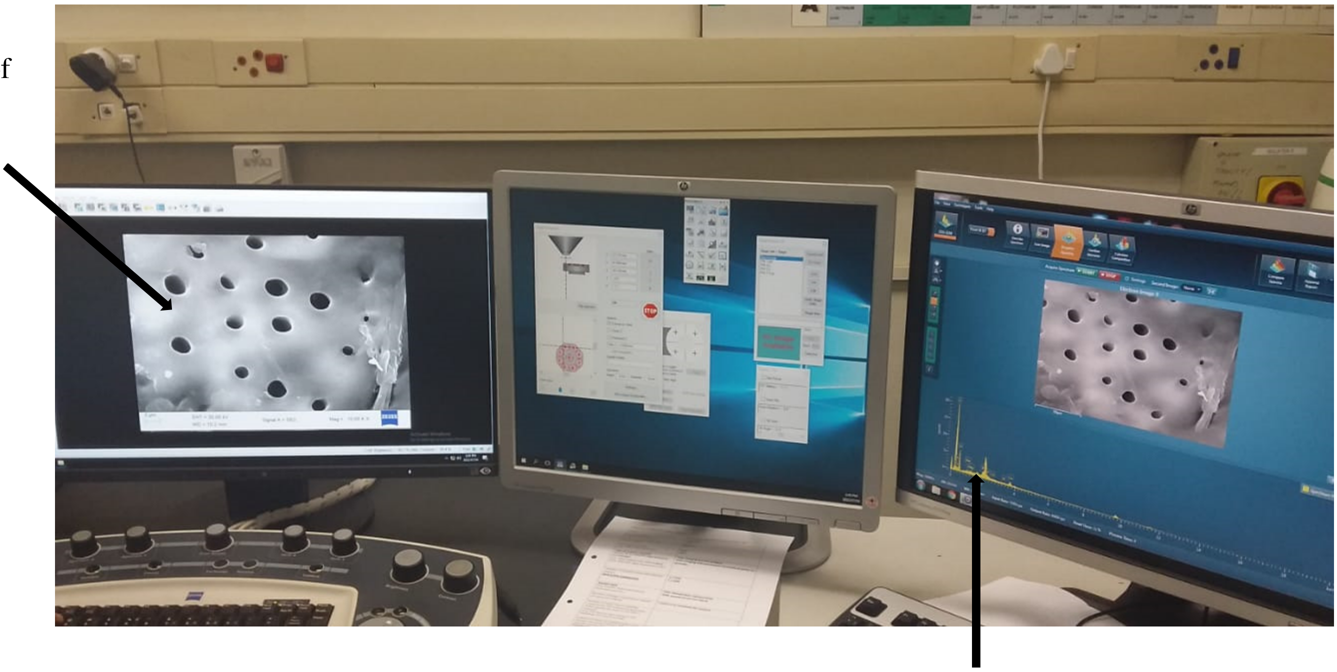
3.4.2.3 Brushing test

Each specimen from the respective groups was brushed with a toothbrush powered with a 1.5v alkaline battery (Oralwise, China) for two minutes and allowed to dry for 30 seconds before rinsing with deionized water. Brushing was performed at room temperature. The slurry of the respective sample powders was prepared by mixing 100 mg of the powder/with 200 µL of deionized water.

3.4.2.4 Scanning Electron Microscope evaluation of the occluded specimen

As illustrated in Figure 3.10, an SEM (field emission, Carl Zeiss) operating in controlled atmospheric conditions at 20 kV was used to evaluate the occluded dentin pre-and post-treatment. Prior to SEM observation, the surface was coated with a thin, electric conductive gold film to prevent a build-up of electrostatic charge. Additionally, the ratios of occluded and opened 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 ×5000 magnification images (n = 5).

Image of
tooth



EDX spectrum

Figure 3. 10: SEM image analysis of the tooth sample

3.4.2.5 Statistical analysis of the occluded area

The mean values of the occluded area ratio in the SEM study were evaluated with one-way analysis of variance (ANOVA) with statistical software (IBM SPSS Statistics v28; IBM Corp), This was followed by a multi-comparison test with Bonferroni correction ($\alpha = .05$).

3.5 VALIDITY AND RELIABILITY

The two most important aspect of experimental research design is centred on the validity and reliability of the data as this ensures the study's credibility. In this study, the internal validity was ensured by following the SANS 1302 (2008) requirement for the preparation, and testing of sampled materials. In addition, the experimental data were further validated using different treatment approaches.

In terms of the study reliability, it is advised that the reliability of an experimental research design be determined by the repeatability and reproducibility of the tests (Walker 2011). Repeatability is the variability of the measurement obtained by one person measuring the same item repeatedly whilst reproducibility by contrast, is the variability of the average values obtained by several observers measuring the same item Slezák and Waczulíková (2011) and Vitek and Kalibera (2011). In this study, the repeatability and reproducibility were assured by the different number of bovine samples used in assessing the occluding characteristics of the sample powders.

3.6 CONCLUSION

In summary, this chapter has exhaustively explained the research approach and the experimental design strategy conducted in the study. The different phase of the research approach was provided as well as the study validity and reliability process. The next chapter provides the results and discussion which were presented based on papers published and or submitted to DHET journals.

Paper I

Onwubu, S.C., Naidoo, D., **Mkhize, S.C.,** Mabaso, N.L.N., Mdluli, P.S. and Thakur, S. 2020. An investigation in the remineralization and acid resistant characteristics of nanohydroxyapatite produced from eggshell waste via mechanochemistry. *Journal of Applied Biomaterials & Functional Materials*, 19 (7): 1-8**.** https://doi.org/10.1177/2280800020968352

JABFM Journal of Applied Biomaterials & Functional Materials

Original Research Article

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Comparative assessment of the remineralization characteristics of nano-hydroxyapatite extracted from fish scales and eggshells

Sandile Cromwell Mkhize, Stanley Chibuzor Onwubu, Thabang Hendrica Mokhothu, Phumlane Selby Mdluli and Ajay Kumar Mishra

Abstract

Objectives: Dentine hypersensitivity (DH) is a common concern in dentistry that has the potential to restrict daily activities and harm a person's quality of life. In this study, the remineralization characteristics of nano-hydroxyapatite (nHAp) extracted from waste eggshells and fish scales were comparatively assessed.

Materials and methods: The extraction methods used to obtain nHAp from both fish scales and eggshells are also described. The effect of the extraction process and bio-waste source on the physicochemical characteristics of the nHAp such as Ca/P ratio, functional groups, crystallinity and phase change, and surface morphology are presented in the study. The remineralization properties were evaluated using dentine models ($n = 15$). A field scanning electron microscope was used to evaluate the effectiveness of the dentine tubules occlusion. The percentage occluded area for all the specimens was evaluated statistically using a one-way analysis of variance ($\alpha = 0.05$).

Results: The results showed that there were variations in the physicochemical characteristics of the nHAp extracted, including the crystallinity, particle size, and surface morphology, and buffering effects against citric acids. The EnHAp extracted from eggshells had higher crystallinity, superior buffering effects, and smaller particle size compared to the nHAp extracted from fish scales, making it a more favourable material for remineralization of teeth. The statistical evidence showed that there were statistically significant differences in the dentine occluding properties measured in the nHAp ($p < 0.001$). The highest mean % occluded area was measured with the EnHAp group.

Conclusions: The findings of this study provide insights into the use of bio-waste materials for the development of sustainable and effective products for oral health care.

Keywords

Bio-waste, eggshells, fish scales, nano-hydroxyapatite, remineralization

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Highlights

- Dentine hypersensitivity (DH) has been a common concern.
- Remineralization characteristics of nHAp extracted from eggshells and fish scales.
- Physicochemical characteristics of the nHAp extraction.

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- EnHAp extracted from eggshells showed higher crystallinity.
- Superior buffering effects and smaller particle size.
- EnHAp shows more favourable material for remineralization of teeth.

Introduction

Recently, the use of bio-based materials has gained popularity and has thus attracted many researchers who aim to explore their potential for biomedical applications.[1,2] The unique properties of these materials such as their non-toxicity, abundant availability, biocompatibility, and biodegradability make them favourable for use in various applications including antimicrobial coatings,[3] drug carriers[4] and adsorbents.[5] Particularly, the healthcare sector has been revolutionized by the use of bio-based materials due to the growing use in a variety of multidisciplinary fields including dentistry.[6] From a dentistry context, the use of bio-based materials has brought about tremendous growth and advantage in dental practice. For example, bio-based materials such as hydroxyapatite have been widely used in preventive and conservative dentistry,[7,8] restorative and periodontal treatment.[9] This makes hydroxyapatite bio-based materials a game-changer in the treatment and management of dental and oral-facial conditions.

In the era of preventive dentistry, the literature suggests periodontal therapy and improved home care practices may result in individuals retaining their teeth for longer, potentially leading to an increase in the number of exposed dentin surfaces.[10] This could result in a condition known as dentine hypersensitivity (DH). DH, a common oral health problem, is defined by the Canadian Association of Dentistry as a ‘short, sharp pain arising from exposed dentin in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical, and which cannot be attributed to any other form of dental defect or pathology’.[11]

In an attempt to manage DH, a variety of topical desensitizing toothpastes, which are dentin tubule occluding materials containing materials like potassium oxalates,[12] strontium salts[13] and sodium fluoride[14] among others. The use of hydroxyapatite particles, particularly in the form of nanohydroxyapatite (nHAp), has emerged as a promising material for remineralizing dentin. This is supported by clinical evidence, which suggests that nHAp-based desensitizing agents have shown promising results in improving DH.[7,8] It is important to note that nHAp is a natural component of the human tooth and bones, composing about ~90% of enamel and ~70% of dentine,[15] which thus makes nano-hydroxyapatite ideal for remineralization of damaged dentine tubules.

In our previous studies, we demonstrated the in vitro extraction of nHAp using eggshell waste as substrate and its use in occluding dentin tubules.[16,17] Fish scales can also be a source of nHAp. Fish scales, which are often considered waste, have been shown to contain valuable inorganic and organic components, including collagen (40%–55%) and hydroxyapatite (15%), making them a useful alternative source for hydroxyapatite extraction.[18,19] Thus, fish scale waste has tremendous unexploited potential as a biomaterial that could potentially add value to waste.

The fish market generates a significant amount of waste, which is either discarded or allowed to rot and represents a major environmental threat by acting as bacterial incubators.[20] These wastes generate large amounts of methane and other harmful gases[21] that pose a significant challenge to the environment. Thus, the creation of valuable, sustainable products from fish waste would serve as a dual-purpose strategy, enabling the extraction of nHAp from a sustainable source while also producing a valuable product. Furthermore, using the fish scale in the extraction of nHAp is important in addressing the environmental problem of waste management and towards a circular economy that supports a cleaner and greener environment. The objective of this study was to evaluate the effectiveness of nHAp derived from fish scales in occluding dentinal tubules.

In this study, we compared the characteristics of nHAp extracted from fish scales with that of eggshells. Fish scale nHAp (FnHAp) was extracted from fish scales in two separate batches. In the first batch, we use common chemicals such as sodium hydroxide (NaOH) and acetic acid in the extraction process. Thereafter, the extracted FnHAp were milled using a planetary ball-milling process. We assess the effectiveness of these materials in the remineralization of dentine tubules using the agitation process proposed by Onwubu et al.[22] To the best of our knowledge, there is little evidence in research that has comparatively compared the effectiveness of natural nHAp extracted from eggshell and fish scale wastes in occluding dentine tubules. The study hypothesized that there will be no significant difference in the occluding abilities of the nHAp extracted from eggshells (EnHAp) and fish scales (FnHAp).

Materials and method

Extraction of nHAp from eggshell and fish scale waste

Collected eggshells and fish scale waste from local outlets in South Africa were processed following the recommendations given by Onwubu et al.[23] These were heated in a furnace for 1 h at 300°C, then heated for 3 h at 900°C at a rate of $3°C\,min^{-1}$ to produce a white powder. This was a result of the calcium carbonate component of the eggshell waste breaking down to yield a calcium oxide powder.[16] Using the stoichiometric ratio of 1:5, 20 g of the white powder and 13.4 g of sodium triphosphate were wet-milled together in a 250 mL bowl containing 100 mL deionized water at 500 rpm for 5 h. The mixture was then centrifuged for 30 min at a speed of 1000 rpm and dried in an oven for 5 days at 40°C. Thirty stainless steel balls with a diameter of 10 mm were used in a planetary ball mill (Retsch® PM 100) during the milling process.

The extraction of nHAp from fish scales was carried out in two batches. In the first batch, the scales were immersed in 1M hydrochloric acid (HCl) for 24 h. After 24 h, they were thoroughly rinsed with reverse osmosis water and then soaked in 1M sodium hydroxide (NaOH) for an additional 24 h to remove proteins. The fish scales were then rinsed and placed in a beaker with water, which was boiled for 20 min on a hot plate at 80°C. The fish scales were dried in an oven at 80°C for 2 h, then placed in crucibles and heated in a furnace at 700°C for 2 h. In the second batch, the obtained power (FnHAp) was dry ball-milled at 500 rpm for 2 h using the same milling parameters as explained above to obtain milled fish scale nHAp (mFnHAp).

Characterization

Fourier transform infrared spectroscopy analysis. The functional groups that were present in the prepared powders were determined using a Perkin Elmer Universal ATR. A background investigation was initially conducted before scanning. A small quantity of the prepared sample powders was then placed in a sample holder and scanned between 400 and 4500 cm^{-1} with a resolution of 4 cm^{-1}.

X-ray diffraction analysis. To track any potential changes in the crystallinity of the prepared powders, an X-ray diffraction (XRD) analysis was conducted. The patterns were captured using a diffractometer (D8 Advance BRUKER AXS instrument Germany instrument); Cu-Ka radiation (lKa1 = 1.5406) the patterns were analyzed between 10 and 80 (2 theta). The parameters used were 40 kV of voltage, 40 mA of current and 0.5 s of pass time.

High-resolution transmission electron microscopic analysis. In a high-resolution transmission electron microscope (HRTEM); (model: Philips CM 120) operating at 120 kV, the size, shape, and distribution of the prepared powders were investigated. Before HRTEM observation, tiny amounts of the powders were each dispersed in 10 mL of ethanol and sonicated at 10 kHz for 10 min. Finally, using a Leica microtome (South Africa), thin cross-sections of cryo-microtomed specimens were created and placed on carbon copper grids measuring 100 mm × 100 mm.

Elemental analysis of samples. The calcium to phosphate ratio of the samples was evaluated using the Energy Dispersive X-ray (EDX) technique. The findings were presented as a percentage weight of all the elements. Due to the interest in estimating the calcium to phosphate ratio that exhibits the component of the tooth's hydroxyapatite, data for carbon, oxygen, phosphorus and calcium are presented.

Table 1. Sample groups.

Sample powder	Total
EnHAp	5
FnHAp	5
mFnHAp	5

n = number of bovine teeth.

pH test. A pH metre (BOECO, BT-675, Germany) with a temperature sensor was used to monitor the pH value. About 1.5 g of each of the sample powders (EnHAp, FnHAp and mFnHAp) was put into a beaker containing 50 mL of deionized water. This mixture was stirred continuously for 10 min at a speed of 600 r min^{-1}, with pH readings taken every minute.

Dentin tubule occlusion test

Recently extracted anterior bovine-enamel teeth (15) were acquired at a slaughterhouse in Durban, KwaZulu Natal, South Africa. The bovine teeth were washed thoroughly and sterilized in a solution of 10% chloroxylenol. Using a low-speed water cooling diamond saw, dentin discs measuring 5 mm × 5 mm × 1 mm were cut below the dentin-enamel junction. The generated dentin disc was then wet-processed for 60 s using silicon carbide polishing sheets (600–1000 grits). Before creating the sensitive tooth model, the dentin discs were placed in a resin (AMT composite, South Africa). Afterwards, to open the dentine tubules, the specimens were submerged for 5 min in a citric acid solution with a concentration of 8 wt%. As shown in Table 1, the specimens were randomly allocated into four groups ($n = 5$) and treated using agitation techniques described by Onwubu et al.[16]

SEM evaluation of the occluded specimen. The occluded dentin before and after treatment was evaluated under controlled air conditions using an SEM (field emission, Carl Zeiss) operating at 20 kV. A thin, electrically conductive gold coating was applied to the surface to prevent an electrostatic charge build-up before SEM observation. With the aid of the ImageJ software (National Institute of Health, USA, http://imagej.nih.gov/ij) the ratios of both the occluded and the opened tubules were calculated. The ratios were determined by dividing the area of blocked tubules by the area of all tubules using 10,000 magnification photos ($n = 5$).

Analysis of the data. To study the mean values of the occluded area ratio in the SEM investigation, a one-way analysis of

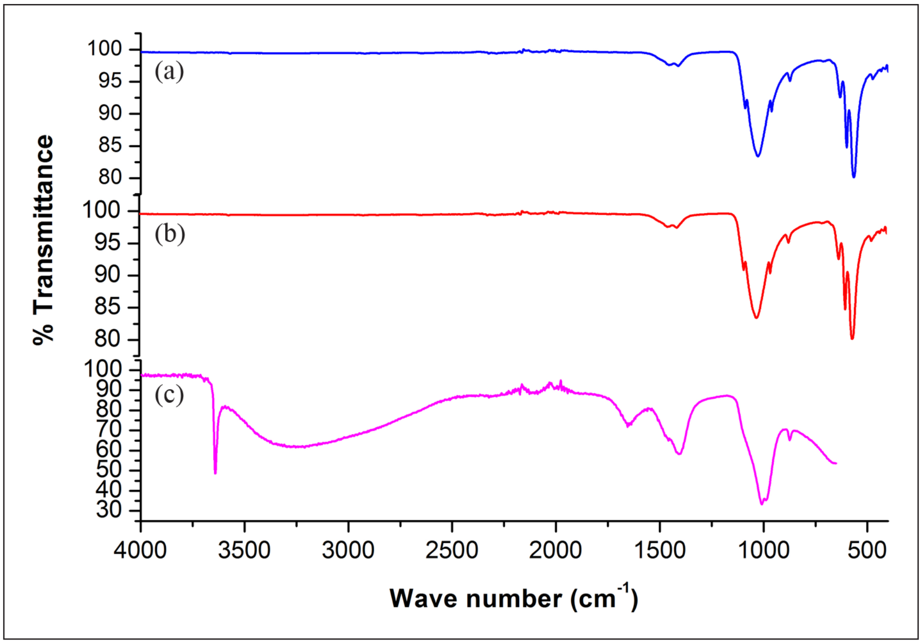

Figure 1. FTIR spectra of: (a) FnHAp, (b) mFnHAp and (c) EnHAp.

variance (ANOVA) was conducted using statistical software (IBM SPSS Statistics v28; IBM Corp). Afterwards, a multi-comparison test with Bonferroni correction (=0.05) was performed. To determine the statistical significance of differences between the groups. The results were considered statistically significant if the *p*-value was less than 0.05. The findings of the SEM investigation were presented as the mean ± standard deviation of the occluded area ratio.

Results

Characterization of the extracted hydroxyapatite samples

The image in Figure 1(a)–(c) shows the characteristic absorption peaks of the extracted nHAp powders as revealed by the FTIR analyses. The absorption bands typical of hydroxyapatite characteristics were observed around ~1000 and ~560 cm^{-1} for all the samples, respectively. This is due to the asymmetrical stretching and asymmetric bending vibrations of the PO_4^{3-} group, as reported in literature.[16,24] While the carbonate bands at 1450 cm^{-1} were evident in all the samples (Figure 1(a)–(c)), the intensity was much more prominent in the EnHAp (Figure 1(c)). The presence of the carbonate group in all the samples is attributable to out-of-plane bending mode and asymmetric stretching of CO_3^{2-} ions in the structure of the nHAp produced.[17] The reason for the carbonate peak found in all the samples may be associated with the substitution of the PO_4^{3-} ions in the structure of nHAp.[24,25] The hydroxyl group (O–H) bending was observed at around ~3750 cm^{-1}, identified by a small absorption peak in samples shown in Figure 1(a) and (b). In contrast, the absorption band for EnHAp (Figure 1(c)) was very visible around 3750 cm^{-1} and it is attributable to the OH stretching.[17] Furthermore, the broadband around ~3450 and 3000 cm^{-1}, and also the sharp band at 1600 cm^{-1} associated with water molecules[16] were evident in EnHAp samples alone (Figure 1(c)). The probable cause for the absence of water molecules in Figure 1(a) and (b) could be related to the alkaline hydrolysis and mechanochemical processes involved in the extraction of nHAp from fish scale.

The image in Figure 2(a)–(c) shows the XRD patterns of the extracted nHAp. The diffraction angles marked at 25.8° (002), 31.8° (121), 32.2° (112), 32.9° (030), 39.8° (310), 46.7° (222) and 49.4° (123), observed in all samples, are indicative of nHAp, according to the international standard (JCP2-76-0694). The peak observed at 29.4° corresponds to calcite, while the peak around 34.1° suggests the presence of Portlandite (20). The crystallinity value of EnHAp (82.19%) was found to be higher when compared to FnHAp (67.48%) and mFHAp (67.22%). The close values in crystallinity between FnHAp and mFnHAp suggest that milling did not impact the crystallinity of the nHAp extracted from fish scales. The Gaussian plot was used to determine the peak area, and the formula below was used to determine the crystallinity.

$$\text{Crystallinity} \quad \frac{\text{Area of crystalline peak}}{\text{Area of all peaks}} * 100$$

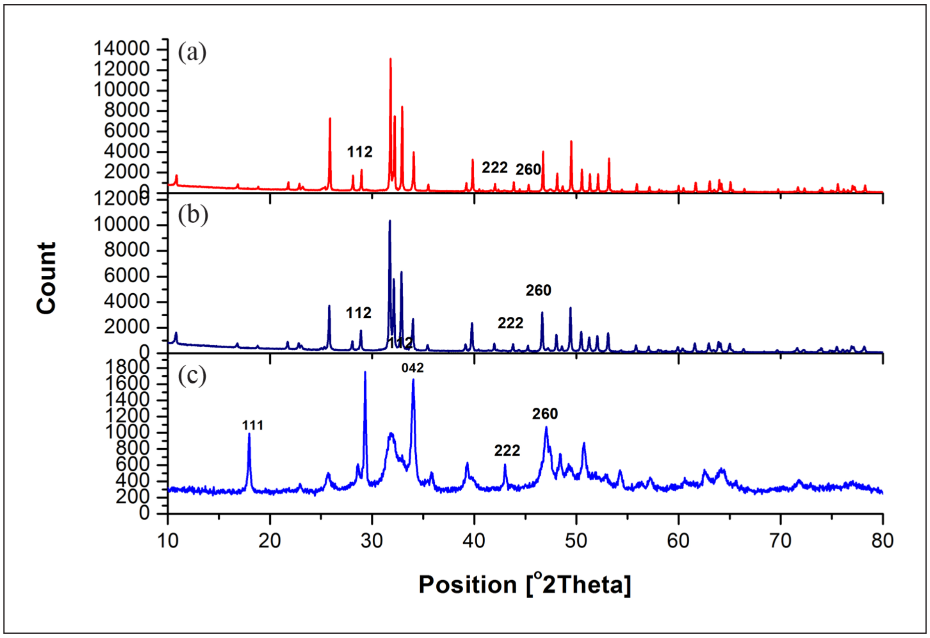

Figure 2. XRD pattern of: (a) FnHAp, (b) mFnHAp and (c) EnHAp.

Table 2. Showing mineral content of the sample powders, as determined by XRD analysis.

Mineral	Chemical formula	FnHAp	mFnHAp	EnHAp
Portlandite	$Ca(OH)_2$	3% (ICDD reference file 96-900-6834)	18% (ICDD reference file 96-100-1788	13.8% (ICDD reference file 98-020-2222)
Calcite	$CaCO_3$	12% (ICDD reference file 96-900-9668	8% (ICDD reference file 96-900-7688	2.5% (ICDD reference file 98-016-4935)
hydroxyapatite	$Ca4(PO4)_6OH_2$	77% (ICDD reference file 96-230-0274)	79% (ICDD reference file 96-230-0274	74.8% (ICDD reference file 96-230-0274)

EnHAp: eggshell nano-hydroxyapatite; FnHAp: fish scale nano-hydroxyapatite; mFnHAp: milled fish scale nano-hydroxyapatite; XRD: X-ray diffraction.

The International Centre for Diffraction Data (ICDD) reference codes were the reference standard used for XRD analyses. These codes guided the identification of organic and inorganic crystalline materials.

The mineralogical content in the different extracted nHAp (FnHAp, mFnHAp and EnHAP) obtained from the XRD analysis is further represented in Table 2. The analysis revealed differences in the mineralogical composition of the key minerals identified by the XRD analysis. The highest composition of hydroxyapatite (79%) and portlandite (18%) were measured in mFnHAp, respectively. By contrast, calcite content was higher in FnHAp (12%) compared to mFnHAp (8%) and FnHAp (2.5%).

The highest concentration of hydroxyapatite in mFnHAp indicates that milling the fish scales improved the mineral content compared to the initial extracted FnHAp. The presence of portlandite in mFnHAp suggests that the milling process could have led to the formation of new phases from the reaction of $Ca(OH)_2$ with CO_2 from the atmosphere. The difference in calcite content between FnHAp and mFnHAp highlights that the milling process might have caused the loss of some of the calcite content during the process. EnHAp showed the lowest content of hydroxyapatite (70%) compared to the other two samples, which suggests that the extraction process using an alkaline solution might have resulted in the loss of some of the hydroxyapatite content. The presence of calcite (2.5%) and portlandite (26%) in EnHAp indicates that the extraction process using an alkaline solution might have led to the formation of new minerals in the sample.

The elements present in the sample groups (FnHAp, mFnHAp and EnHAp) were analyzed by EDX and the results are shown in Table 3. The elements found in the samples were carbon (C), calcium (Ca), oxygen (O), phosphorus (P) and sodium (Na) with the presence of sodium being only evident in mFnHAp and EnHAp. All sample groups had high levels of calcium and phosphorus in the form of carbonate and phosphate. The ratio of calcium to phosphorus (Ca/P) was found to be different for each sample, with mFnHAp having a ratio of 1.9, slightly higher

Table 3. Elemental analysis of the samples.

Element (weight in %)	Sample groups		
	EnHAp	FnHAp	mFnHAp
Carbon (C)	17.5	10.5	15.3
Calcium (Ca)	26.9	66.3	21.4
Oxygen (O)	39	15.6	45
Phosphorus (P)	13	7.9	11.1
Sodium (Na)	3.0	0	4.8
Magnesium	0.6	0	2.4
Ca/P	2.1	2.1	1.9

than the 1.67 ratio found in human bones and teeth. The ratios for FnHAp and EnHAp were slightly different from the stoichiometric ratio of 1.67, which is likely due to the presence of trace elements like sodium and magnesium in natural nHAp.[26,27]

The HRTEM image in Figure 3(a)–(c) shows the surface morphology of the sample powders. There were visible differences in the surface morphology of the nHAp extracted from eggshells and fish scales. The image in Figure 3(a) and (b) shows that the nano-hydroxyapatite from fish scales (FnHAp and mFnHAp) particles presented an irregular morphology. After milling at 500 rpm for 2 h, the mFnHAP (Figure 3(b)) showed an increase in surface smoothness in comparison with FnHAp (Figure 3(a)). By contrast, an irregular-rod-like structure was observed for the eggshell nano-hydroxyapatite (EnHAp). The particles also appeared to assemble into short chains that stuck together into clusters (Figure 3(c)). In addition, the particle size of the sample powder measured using Image J software showed that the EnHAp had a mean diameter of 11.6 ± 2.7 nm. By contrast, the mean diameter measured for FnHAp and mFnHAp were 190 ± 81.8 and 74.6 ± 33.7, respectively.

Figure 4 shows the pH of the sample powders in deionized water and citric acid solution with initial pH of 2.06. The findings indicated that EnHAp had the highest mean pH value in deionized water (13.09), whereas the mFnHAp had the lowest value (10.77). All the sample powder had alkaline characteristics that may be attributable to the elevated calcium content. EnHAp had the best buffering characteristics when compared to both FnHAp and mFnHAp from fish scales in the sample powder study using the citric acid solution at pH 2.06 since it effectively neutralized the pH of the citric acids (Figure 4).

Additionally, it was also noted that the EnHAp showed the greatest buffering capacity as the slope of the EnHAp was the smallest among the sample groups. The results showed that EnHAp was more effective in maintaining the stability of the pH in the citric acid solution compared to FnHAp and mFnHAp. The ability to buffer pH is important in several biological processes and the results suggest that EnHAp may have potential applications in the biomedical field.

Dentine tubule occluding characteristics

FESEM was used to examine the morphological alterations of the occlusion of the dentine tubule before and after treatment (agitation). The samples treated with EnHAp (Figure 5c2) and mFnHAp (Figure 5b2) had more occluded areas than the samples treated with FnHAp (Figure 5a2). All samples show visible evidence of dentine tubule occlusion after agitation. The nanoparticles can be seen on the surface of the dentine specimens, and it can be inferred that the nanoparticles have entered the dentine tubules and have caused the occlusion. The results indicate that the EnHAp and mFnHAp were more effective in occluding the dentine tubules compared to FnHAp, which could be due to the difference in size and surface morphology of the particles. The smaller size and the rod-like structure of the EnHAp particles might have allowed for easier penetration into the dentine tubules, leading to more effective occlusion. On the other hand, the irregular shape of the FnHAp particles might have hindered their penetration into the tubules, resulting in less effective occlusion.

The mean % occluded area of the dentine tubules for the treated specimens is given in Table 4. The ANOVA value shows that the total mean % ratio of the dentine tubules occluded area for the specimens treated with sample powders (EnHAp, FnHAp and mFnHAp) was statistically significant ($p < 0.001$). The results indicate that EnHAp had the highest % mean occluded area (78.2 ± 1.5%) while the lowest was measured for the FnHAp group (59.7 ± 1.7%). According to the results of the Bonferroni test, the percentage of tubules occluded for the EnHAp group was statistically higher than that of the FnHAp group and the mFnHAp group ($P = 0.002$ and 0.001, respectively). In comparison to the FnHAp group, the % occluded area measured for the mFnHAp group was higher ($p = 0.001$).

Discussion

The clinical condition of DH has the potential to impede and restrict daily activities, which negatively impacts the person's quality of life.[28] According to Balhuc et al,[29] the use of more complex products and combinations between nano-hydroxyapatite (nHAp) and various compounds is an innovative method for significantly reducing DH and remineralizing dentinal tubules. While synthetic nHAp is commonly used in biomaterial applications, the need for environmental sustainability products has created a market for bio-waste materials. In this present study, we comparatively assessed the remineralization characteristics of nHAp extracted from waste eggshells and fish scales. The

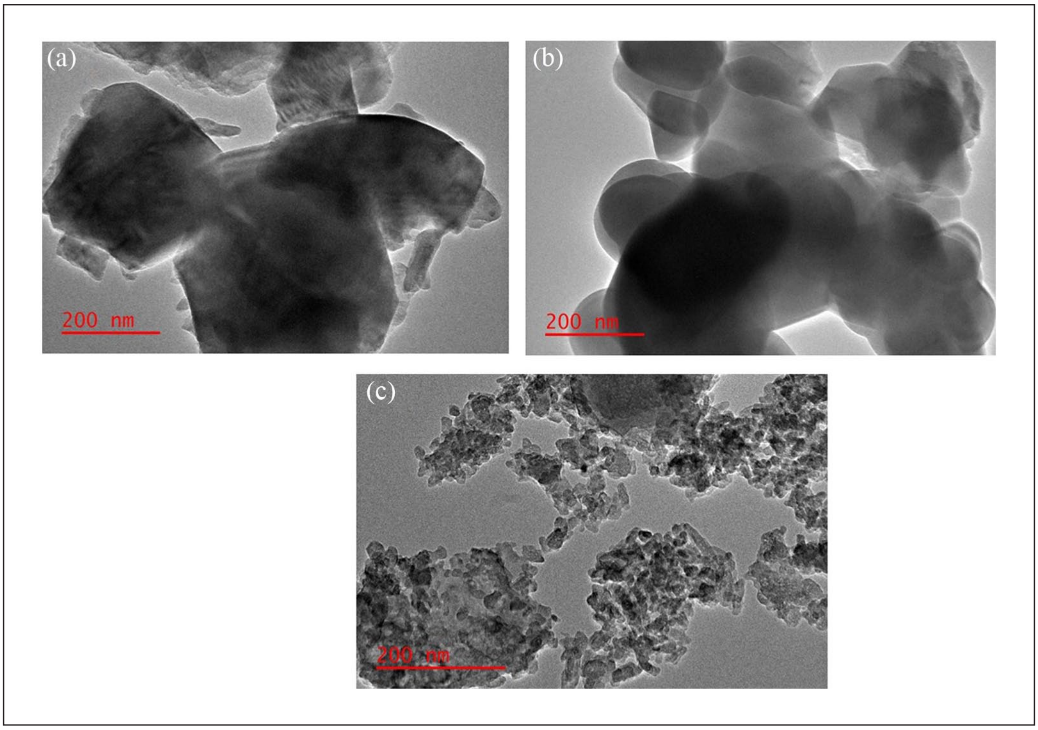

Figure 3. HRTEM micrograph of: (a) FnHAp, (b) mFnHAp and (c) EnHAp.

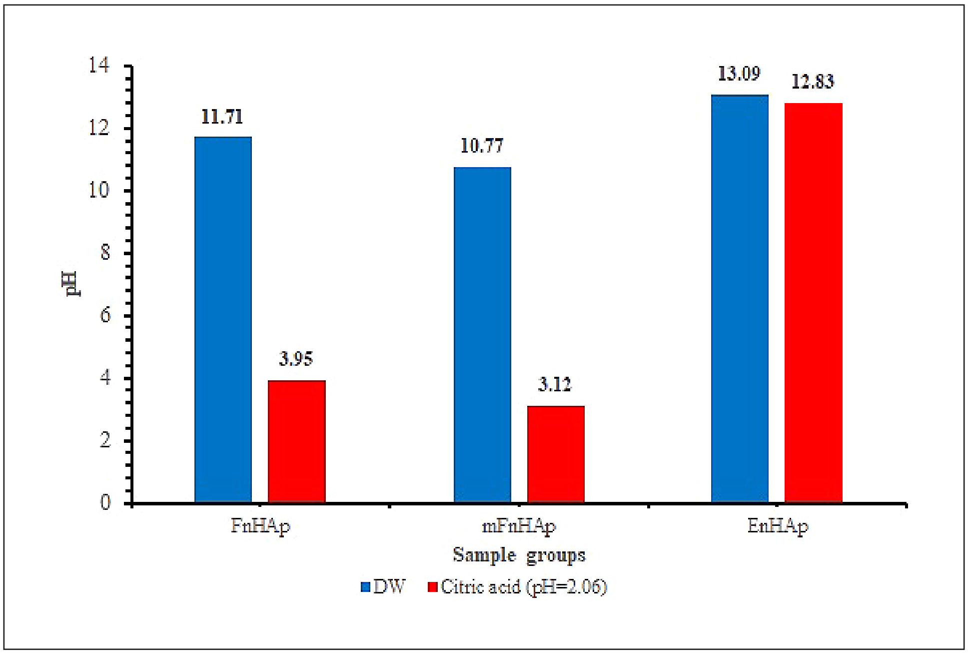

Figure 4. Buffering characteristics of samples.

results showed slight variations in the physicochemical characteristics of the nHAp extracted (Figure 1). While the nHAp from eggshells had higher crystallinity, the mineralogical content of hydroxyapatite was higher in the fish scale nHAp (Table 2).

The surface morphology also revealed variations between the nHAp extracted from eggshells and fish scales (Figure 3). The study results showed that milling the nHAp extracted from the fish scale does not negatively impact the physicochemical characteristics, but the milled fish

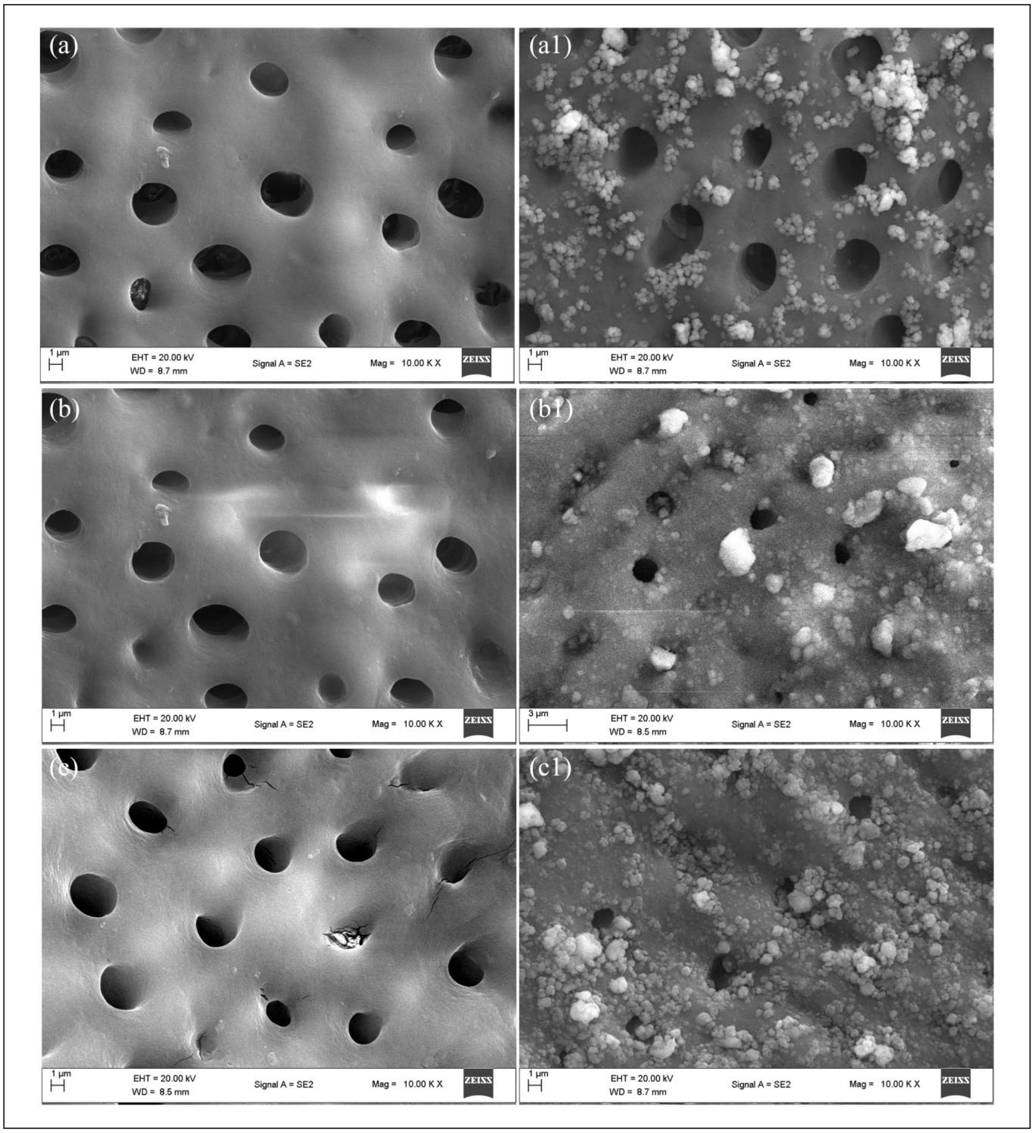

Figure 5. Remineralization test: (a) FnHAp, (b) mFnHAp and (c) EnHAp (1 represents after agitation).

scale nHAp (mFnHAp) had a smoother surface morphology and reduced particle size after milling (Figure 3). This may be due to the effect of the mechanical energy during milling, which could have triggered a transition from aggregated FnHAp to a smoother mFnHAp (Figure 3). Another study reported that milling stimulates transitions of substances due to the impact of mechanical energy.[30] Based on the study results, it can reasonably be assumed that the physicochemical characteristics of the nHAp such as the crystallinity, particle size and surface morphology vary due to the source of bio-waste, method of extraction, pH and temperatures.[31]

Furthermore, the results indicate that the buffering characteristics of eggshell nHAp (EnHAp) against citric acids were superior to those measured for nHAp extracted from fish scales (Figure 4). The reason for EnHAp having higher buffering effects compared to FnHAp and mFnHAp could be due to the differences in the crystal structure and particle size. The irregular rod-like structure and smaller particle size of EnHAp may provide a larger surface area for reaction with the citric acid, allowing it to effectively neutralize the pH of the solution. The higher surface area could lead to a greater number of surface-active sites, resulting in more efficient buffering effects. It is also possible that differences in the chemical composition and the presence of trace elements in each sample group could play a role in the buffering characteristics of the materials. This indicates that EnHAp provides better protection against enamel demineralization from acidic substances by effectively neutralizing citric acid. The implication for oral health are that EnHAp provides high bioavailable calcium that is crucial for tooth remineralization.[17,32] This is

Table 4. ANOVA test comparison of the occluded area.

Treatment group	*N*	Mean ± SD	Standard error	95% confidence interval		*p*-Value	Bonferroni's correction
				Lower bound	Upper bound		*p*-Value
EnHAp	5	78.2 ± 1.5	0.680	76.332	80.108	<0.001	$0.002^{1,3}$
FnHAp	5	59.7 ± 1.7	0.753	57.629	61.811		$0.001^{1,2}$
mFnHAp	5	72.9 ± 2.1	0.939	70.332	75.548		$0.001^{3,2}$

EnHAp: eggshell nanohydroxyapatite; FnHAp: fish scale nanohydroxyapatite; mFnHAp: milled fish scale nanohydroxyapatite; SD: standard deviation. The superscript numbers indicate the mean differences between sample groups.

supported by the FESEM image in Figure 5, which shows that EnHAp had the most increase in dentine tubules occlusion. In agreement with Tschoppe et al,[33] this means that the higher pH values of the nHAp slurries favour tooth remineralization. Statistical results indicate a statistically significant difference in the treated dentine specimens ($p < 0.001$), and the formulated study hypothesis was rejected based on the results obtained.

Overall, the EnHAp group had the highest mean % occluded area of the dentine tubules (Table 4). The plausible difference in the dentine tubules observed may be due to the physicochemical characteristics of the nHAp extracted. This aligns with Kong et al[34] who suggest that the performance of nHAp is influenced by its crystallinity, particle size, and surface morphology. This means that the smaller particle size of EnHAp and the higher pH values favour its remineralization characteristics over the fish scale nHAp. Additionally, the particle sizes measured for the nHAp extracted are smaller than the size of tubules close to the dentine-enamel junction, which is around 3–5 µm.[35] This indicates that smaller nHAp particles can easily be attached to dentinal surfaces and occlude exposed dentine tubules. This may explain the dentine tubule occlusion in all the tested nHAp from both fish scales and eggshells (Figure 5). Another possible reason is the polarity and the large surface proportion of the atomic number of nHAp, which allows the particles to bind to collagen and hydroxyapatite in dentine.[36]

Conclusions

The study we have compared the remineralization characteristics of nano-hydroxyapatite (nHAp) extracted from waste eggshells and fish scales. The results showed that there were variations in the physicochemical characteristics of the nHAp extracted from the two sources. The EnHAp extracted from eggshells had higher crystallinity and superior buffering effects against citric acids compared to the nHAp extracted from fish scales. The smaller particle size and higher pH values of EnHAp favoured its remineralization characteristics. The results showed that EnHAp had superior buffering effects against citric acids and higher mean % occlusion of dentine tubules compared to FnHAp and milled FnHAp (mFnHAp). The ANOVA results also indicated that there was a statistically significant difference ($p < 0.001$) in the dentine tubule occlusion between EnHAp and FnHAp. These findings highlight the potential of nHAp from natural bio-waste as a low-cost alternative for dental applications and warrant further research to optimize its use in clinical practice.

Declaration of conflicting interests

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Data availability statement

On reasonable request, the corresponding author will provide the datasets generated during and/or analysed during the current study.

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Paper II

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Research Article

An In Vitro Assessment of the Acid Resistance Characteristics of Nanohydroxyapatite/Silica Biocomposite Synthesized Using Mechanochemistry

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This paper reports on the in vitro assessment of the acid resistance characteristics of mesoporous silica/nanohydroxyapatite (MSN@nHAp) biocomposite synthesized through the mechanochemical method. Bovine enamel models were used to study the acid resistance characteristics of the composite ($n = 5$). X-ray diffraction and Fourier transform infrared spectroscopy were used to characterize the surface morphology of the MSN@nHAp. The XRD and FTIR results confirmed the successful syntheses and surface modification of nanohydroxyapatite with silica. The MSN@nHAp exhibits superior acid resistance characteristics. The salient aspect of this study suggests that mechanochemistry is a useful technique in the synthesis and surface modification of valuable biomaterials. The study concludes that the MSN@nHAp composite could be utilised in toothpaste formulation for oral healthcare management due to its acid resistance properties.

1. Introduction

Dentin hypersensitivity (DH) is a noticeable dental problem with a negative consequence on the individual's quality of life [1]. From an epidemiological context, Zeola et al. [2] revealed that discomfort from dentin hypersensitivity is a common finding in the adult population, ranging between 11.5 and 35.5%. While different dentin hypersensitivity toothpastes are commercially available in the market, their overall effectiveness is limited in an acidic environment [3]. Owing to this, a novel approach in the treatment and management of DH is the use of various nanomaterials [4, 5]. Among these materials, mesoporous silica (MSN) and nanohydroxyapatite (nHAp) had gained enormous research interest due to their antibacterial action; physical, mechanical, and biological characteristics; and distinctive particle size [6, 7].

Previous studies reported that nHAp could repair tooth enamel [8, 9], which suggests that it might be useful to treat DH. Besides, nHAps could easily penetrate the dentinal tubules, which may improve their dentin occlusion properties [10]. On the other hand, MSNs had strong osteoconductivity and bioactivity properties [11], which render them compatible with other bioactive materials [12]. In addition, MSNs have been widely used as a dentin tubule occluding material in combination with other inorganic nanoparticles. For example, Tian et al. [12] showed that a new MSN-based biocomposite successfully occludes dentin tubules. Yu et al. [6] reported that the mesoporous silica/hydroxyapatite composite was effective against acidic substances in occluding dentin tubules.

While MSN@nHAp biocomposites are widely synthesized in the laboratory through the precipitation process [13], nevertheless, the chemical method of synthesizing the composite may be cumbersome and time-consuming and, in most times, requires the use of toxic chemicals [14]. Consequently, there is a need for an alternative environmentally friendly technique for synthesizing the biocomposite using the mechanochemical method. The application of the mechanochemical method in biocomposite material preparation has gained widespread interest among researchers, due to its simplicity and environmental friendliness [15]. The technique utilises mechanical force to affect chemical reactions as well as structural changes in a material [16]. Despite the enormous potential of the mechanochemical method, there is limited evidence in its use for the preparation of mesoporous silica and nanohydroxyapatite biocomposites (MSN@nHAp). In this present study, we reported on the use of a mechanochemical method for synthesizing MSN@nHAp. The technique entails wet-milling calcined eggshell waste and sodium triphosphate to form nHAp. Thereafter, the synthesized nHAp was modified with MSN silica by ball milling both materials together using the mechanochemical method. This study was aimed at assessing in vitro the acid resistance characteristics of MSN@nHAp biocomposite synthesized through the mechanochemical method for its potential application in the management of DH.

2. Material and Methods

2.1. Preparation Nanohydroxyapatite and Silica Composite (MSN@nHAp). Locally sourced chicken eggshells were prepared according to the method described by Onwubu et al. [17]. The oven-dried eggshells were calcined by heating them in a furnace at 800°C for 3 hours at a heating rate of 3°C/min. Thereafter, nanohydroxyapatite was synthesized and subsequently modified with mesoporous silica following two steps: step 1: nHAp synthesis through wet-milling. In this step, 20 g of the calcined eggshell powder was wet-milled together with 13.4 g of sodium triphosphate in 100 mL of deionized water using a planetary ball mill (Retsch® PM 100) at 500 rpm for 5 hours. After milling, the mixture was centrifuged at speed of 1000 rpm for 30 min and thereafter oven-dried at 40°C for 5 days. Next is step 2: synthesis of MSN@nHAp through dry milling. Using the planetary ball mill in step 1, mesoporous silica (0.9 μm; Sigma-Aldrich) was used to modify the synthesized nHAp at 500 rpm for 40 min in a ratio of 1 : 10 (1 g of MSN to 10 g of nHAp). The parameters used for the milling process in the two steps include 30 stainless steel balls of 10 mm diameter in a 250 mL bow. The powder obtained after milling was characterized to establish the formation of nHAp in the first step and the subsequent modification with silica in the second step. The particle size and shape of the prepared nHAp are reported in another study [18]. The pH of the samples was measured using a pH meter. 1 g of each sample was dissolved in 30 mL deionized water, and the solution agitated at a low speed of 500 rpm for 1 min. The pH reading measured for the MSN was 2.5, nHAp was 13.94, and MSN@nHAp was 13.07.

2.1.1. Fourier Transform Infrared Spectroscopy Analysis. A Perkin Elmer Universal ATR was used to study the chemical composition and functional group present in nHAp and MSN@nHAp. An initial background check was performed before scanning the samples. Thereafter, few quantities of the samples were placed in the sample holder for FTIR analysis. The scanning range was 400-4500 cm^{-1} at a resolution of 4 cm^{-1}.

2.1.2. X-Ray Diffraction Analysis. The changes in the crystallinity of nHAp, MSN@nHAp, and MSN were studied using an XRD diffractometer (D8 Advance, BRUKER AXS instrument, Germany instrument; Cu-Ka radiation (lKa1 = 1.5406 Å). The samples were analysed between 0 and 90 (2 theta) while the voltage, current, and pass time were kept at 40 Kv, 40 mA, and 0.5 s, respectively.

2.2. Acid Resistance Test on Tooth Enamel. In line with the procedure demonstrated by Onwubu et al. [18], bovine tooth enamel was used to assess the acid resistance characteristics of the samples. The tooth enamel specimens were assigned randomly into five groups ($n = 5$). The tooth enamel was placed in a 4% citric acid solution containing each of the sample powders for 2 min. The images of the specimens before and after acidic exposure were studied using a scanning electron microscope (Field Emission-Carl Zeiss). Energy-dispersive spectroscopy was further used to quantify the elemental loss in the samples after acidic exposure. Each tooth sample was tested at four separate locations on the tooth surface, and the mean elemental loss calculated as a percentage of the total weight of all elements found after acidic exposure.

3. Results and Discussion

3.1. Characterization of the Prepared Composite. The FTIR spectra of the prepared nHAp and MSN@nHAp are given in Figure 1. In Figure 1(a), the spectrum observed around ~1000 cm^{-1} is attributed to the P–O asymmetrical in the hydroxyapatite [19]. The broad spectrum observed around ~3450 cm^{-1} and 3000 cm^{-1} and a sharp peak at 1600 cm^{-1} was attributed to water molecules [7, 18, 20]. The sharp spectrum around 3750 cm^{-1} is attributed to the OH stretching [19]. The absorption bands of carbonates (CO_3^{2-} ions) in the hydroxyapatite structure were observed around 1450 cm^{-1} [7, 18]. On the contrary, the image in Figure 1(c) reveals the presence of Si-O-Si stretching. The presence of Si-O-Si vibration suggests the formation of nHAp crystals within the MSN structure and agrees with other studies [6, 21]. Although Yu et al. [6] in their study observed OH bending in the MSN@nHAp structure, the image in Figure 1(c), however, reveals the absence of OH bending. The plausible explanation for this may be attributed to the dry milling process used in the modification of MSN and nHAp as against the wet-chemical process reported by [6].

Figure 2 depicts the XRD patterns of the prepared nHAp, MSN@nHAp, and MSN. In Figure 2(a) (A), the pattern with (111), (112), (042), and (260) corresponds to the hydroxyapatite peak. This was further confirmed by the international standard (JCP2-76-0694). For the MSN image in Figure 2(a) (C), noncrystalline scattering observed between 10 and 35° is indicative that the material is amorphous [6]. The MSN@nHAp displays peaks, which are

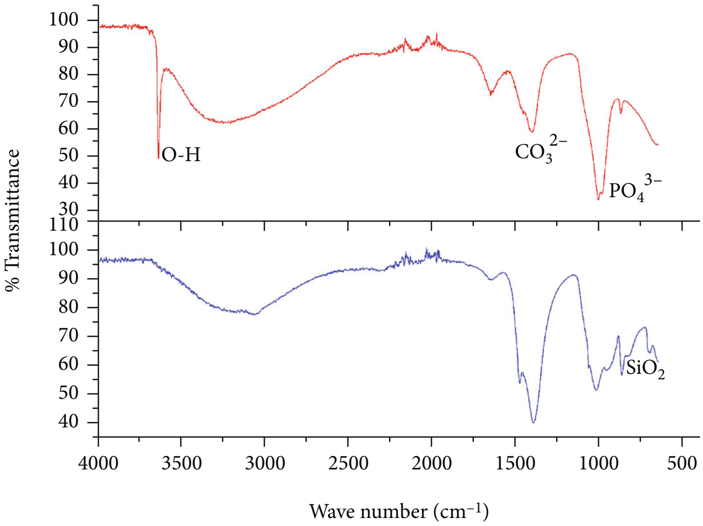


Figure 1: FTIR spectra of (a) nHAp and (b) MSN@nHAp.

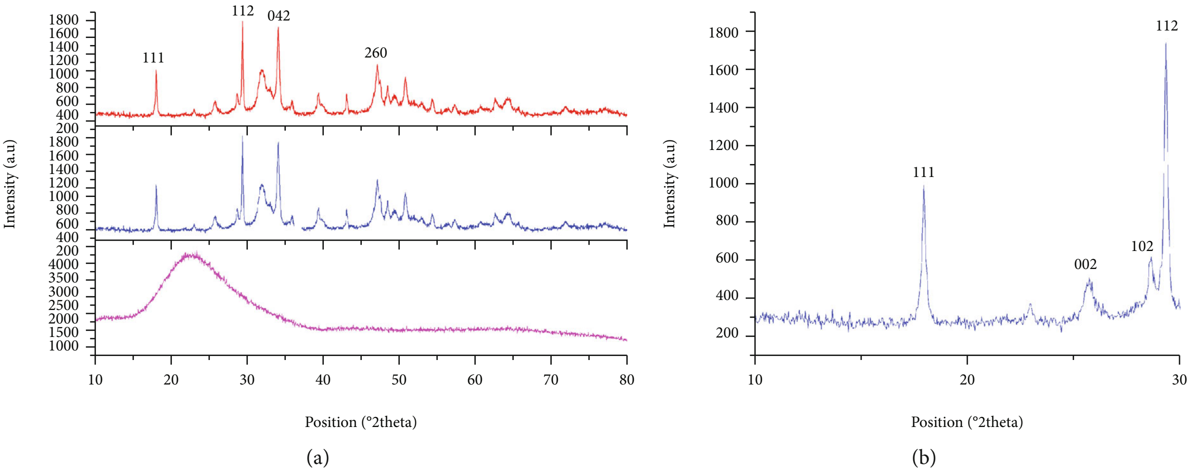


Figure 2: XRD pattern of (a): (A) nHAp, (B) MSN@nHAp, and (C) MSN; (b) extended MSN showing the amorphous region observed between 10 and 15°.

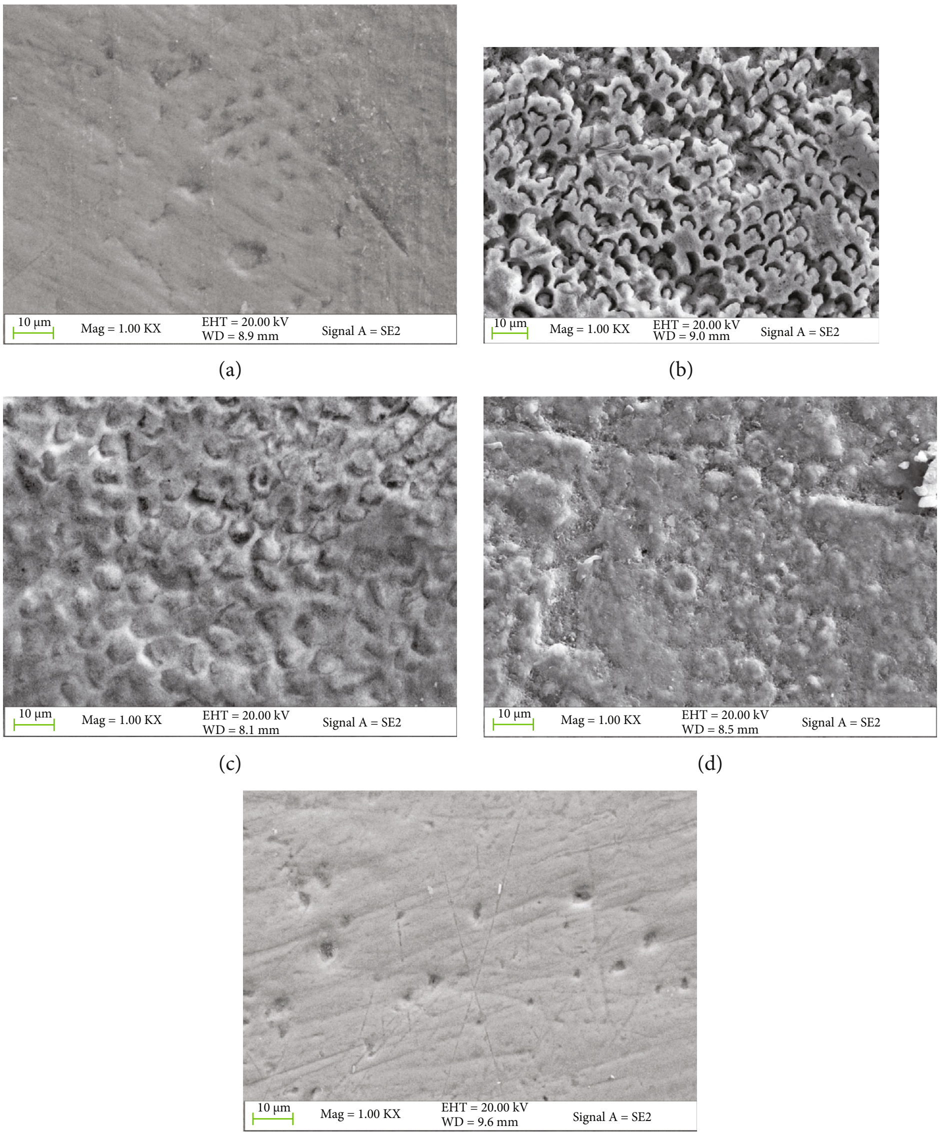
characteristics of the standard diffraction peaks of the nHAp phase and are consistent with the image in Figure 2(a). Moreover, the MSN@nHAp composite shows an amorphous peak which corresponds to the silica peak between 10 to 15° (Figure 2(b)).

The tooth enamel is mainly made up of hydroxyapatite in the form of calcium (Ca^{2+}) and phosphate ions (PO_4^{3-}). It has been suggested in the literature that the loss of Ca^{2+} and PO_4^{3-} ions is indicative of tooth demineralization [22]. The elemental analysis of the samples in Table 1 suggests that the samples exposed to citric acid alone had the most elemental loss of calcium (33.55%) and phosphorus (14.33%) while the nHAp and MSN@nHAp group the lowest elemental losses, respectively. Arguably, it could be assumed that the lower elemental loss of Ca^{2+} and PO_4^{3-} ions in the MSN@nHAp group suggests resistance to acidic attacks while the higher loss of the elements is indicative of enamel demineralization. This is in agreement with Shellis et al. [22] that the exposure of tooth enamel to acidic attacks causes the enamel to release Ca^{2+} and PO_4^{3-} ions to the oral environment to attain a new state of equilibrium.

The FESEM images of the bovine enamel before exposure, exposed to acid alone, MSN, nHAp, and MSN@nHAp, are illustrated in Figure 3. There was visible evidence of the destruction of the prismatic enamel structure in the samples exposed to citric acid alone (Figure 3(b)). This supports the assertion that dietary acids such as citric acid result in enamel demineralization [23]. In Figure 3(c), the MSN, to some extent, offers protection against the erosive challenge. This may be attributed to the acid-resistant stability of MSN, which offers strong acid resistance properties [6]. Furthermore, the FESEM images in Figure 3(d) suggest that nHAp offers effective protection against acid dissolution. There was minimal effect on the enamel surface, which

Table 1: Elemental analysis of mineral loss in tooth enamel samples.

Element (weight in %)	Sample groups				
	Unexposed tooth	Exposed to citric acid alone	MSS	nHAp	MSN@nHAp
Carbon (C)	27.48%	8.54%	13.61%	32.08	28.93
Calcium (Ca)	20.95%	33.55%	30.48%	21.91	21.77
Oxygen (O)	39.42%	42.59%	40.38%	34.41%	35.35
Phosphorus (P)	11.20%	14.33%	14.26%	10.29%	10.71%



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

Figure 3: SEM image of tooth enamel: (a) unexposed tooth; (b) exposed alone; (c) MSS; (d) nHAp; (e) MSN@nHAp.

could be attributed to the pH of the nHAp slurry (pH = 3.94). Consistent with Tschoppe et al. [24], it could be assumed that the higher pH value of the nHAp slurries acts by reducing demineralization.

The FESEM image of the MSN@nHAp suggests that there was outstanding protection against erosive challenges. The image indicates that acid resistance protection of MSN@nHAp was superior when compared to the MSN and nHAp groups, as there was no visible evidence of enamel demineralization. This is in agreement with the finding of Yu et al. [6] who observed similar superior acid-resistant stability for the mesoporous silica and nanohydroxyapatite composites. A plausible explanation for the outstanding enamel protection offered by the composite may likely be due to the capacity of the silica constituent of the composite to increase the bioavailability and slow the release of the incorporated calcium and phosphate ions [25, 26].

4. Conclusion

In summary, the results obtained from the study indicate that MSN@nHAp was successfully synthesized and modified using the mechanochemical method. The FTIR and XRD results confirmed the presence of both crystalline and amorphous structures in the synthesized composite. The FESEM and EDX results indicate that the modified biocomposite exhibits superior acid resistance properties which suggest that it could be useful as a biomaterial for dental application. Particularly, the biocomposite may be highly useful in toothpaste formulation for the treatment of dentin hypersensitivity as well as valuable where an acid resistance characteristic is required in oral care products. Hence, future studies will evaluate the dentin tubule occluding properties of the composite in the management of DH.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Manuscript I

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Comparative assessment of the remineralization characteristics of Nano-hydroxyapatite extracted from fish scales and eggshells

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Abstract

Objectives: The need for environmentally sustainable products has created a niche for bio-waste materials. This study comparatively assessed the remineralisation characteristics of nano-hydroxyapatite (nHAp) extracted from waste eggshells and fish scales.

Materials and Methods: The extraction methods used to obtain nHAp from both fish scales and eggshells are also described. The effect of the extraction process and bio-waste source on the physicochemical characteristics of the nHAp such as Ca/P ratio, functional groups, crystallinity and phase change, and surface morphology are presented in the study. The remineralisation properties were evaluated using dentin models (n=15). Fifteen of the specimens were agitated in a solution of 1g powder and 40mL water for 1 hour, namely: Group 1; FnHAp, Group 2; mFnHAp; and Group 3; EnHAp (n = 5). A field scanning electron microscope was used to evaluate the effectiveness of the dentin tubules occlusion. The percentage occluded area for all the specimens was evaluated statistically using a 1-way analysis of variance ($\alpha = 0.05$).

Results: The characterisation results showed a slight variation in the physicochemical characteristics of the extracted nHAp between eggshell (EnHAp) and fish scales (FnHAp and mFnHAp). The statistical evidence showed that there were statistically significant differences in the dentin occluding properties measured in the nHAp ($p<0.001$). The highest mean % occluded area was measured with the EnHAp group.

Conclusions: From a dentistry context, the study provides a benchmark for further advanced studies on utilising natural nHAp in remineralizing dentin.

Keywords: Bio-waste, eggshells, fish scales, nano-hydroxyapatite, remineralization

Introduction

In recent times, the use of bio-based materials has gained popularity and has thus attracted several researchers that aim to explore their potential for biomedical applications [1, 2]. The unique properties of these materials such as their non-toxicity, abundant availability, biocompatibility, and biodegradability, physical, chemical, and structural properties favour their use in various applications including antimicrobial coatings [3], drug carriers [4], adsorbents [5]. Particularly, the healthcare sector has been revolutionised by the use of bio-based materials due to the growing uses in a variety of multidisciplinary fields including dentistry [6]. From a dentistry context, the use of bio-based materials has brought about tremendous growth and advantage in dental practice. For example, bio-based materials such as hydroxyapatite have been widely used in preventive and conservative dentistry [7, 8], and restorative and periodontal treatment [9]. This, thus, makes hydroxyapatite bio-based materials a game-changer in the treatment and management of dental and oral-facial conditions.

Particularly, in the era of preventive dentistry, it is speculated in the literature that due to periodontal therapy and home care practices, more individuals will keep their into later age; which might result in a greater number of exposed dentin surfaces [10]. This may consequently result in a dental condition known as dentin hypersensitivity (DH). DH is a common oral health problem faced in dentistry and defined by the Canadian Association of Dentistry as a “short, sharp pain arising from exposed dentin in response to stimuli typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other form of dental defect or pathology” [11].

In an attempt to manage DH, numerous dentin tubules occluding materials in the form of topical desensitising toothpaste containing materials such as potassium oxalates [12], strontium salts [13], and sodium fluoride [14] among others are on the market. However, the use of hydroxyapatite particles, particularly in the nano-size has emerged as a promising material for remineralising dentin. This is supported by clinical evidence where nano-hydroxyapatite (nHAp) based desensitising agents have shown promising clinical improvements in DH [7, 8]. Equally important, nHAp is a natural component of the human tooth and bones making up about ~90% of the enamel and ~70% of the dentin [15], which thus makes nano-hydroxyapatite idea for remineralisation of damaged dentin tubules.

In our previous studies, we demonstrated in vitro the extraction of nHAp from eggshell waste and its application in occluding dentin tubules [16, 17]. Another natural waste material that nHAp could be extracted from is fish scales. While fish scales are generally considered worthless, impractical, and dismissed as waste, however, studies have shown that fish scales contain numerous valuable inorganic and organic such as collagen (40-55%) and hydroxyapatite (15%) which makes a useful alternative for the extraction of hydroxyapatite [18, 19]. As such, fish scale waste has tremendous but unexploited biomaterial that could potentially add value to waste.

The fish market generates a significant amount of waste, which is either thrown away or allowed to decay and represent a huge threat to the environment by acting as bacterial incubators [20]. These wastes generate huge amounts of methane and other environmental foe gases [21] that pose a significant challenge to the environment. Thus, the creation of valuable sustainable products from fish waste would serve as a dual-purpose strategy that would enable the extraction of nHAp from a sustainable source while also producing a valuable product. Furthermore, using the fish scale in the extraction of nHAp is vital in the environmental problem of waste management. This will also help towards achieving a circular economy, which is high towards a cleaner and greener. The objective of this study was to assess the efficacy of nHAp derived from fish scales in occluding dentinal tubules.

In this study, we compared the characteristics of nHAp extracted from fish scales with that of eggshells. Fish scale nHAp (FnHAp) was extracted from fish scales in two separate batches. In the first batch, we use common chemicals such as sodium hydroxide (NaOH) and acetic acid in the extraction process. Thereafter, the extracted FnHAp were milled using a planetary ball-milling process. We assess the effectiveness of these materials in the remineralization of dentin tubules using the agitation process proposed by Onwubu, Mdluli [22]. To the best of our knowledge, there is little evidence in research that has comparatively compared the effectiveness of natural nHAp extracted from eggshell and fish scale wastes in occluding dentin tubules. We, therefore, hypothesised that there will be no significant difference in the occluding abilities of the nHAp extracted from eggshells (EnHAp) and fish scales (FnHAp).

Materials and Method

2.1 Extraction of nHAp from eggshell and fish scale waste

Collected eggshells and fish scale waste from local outlets in South Africa were processed in accordance with the recommendations given by Onwubu, Vahed [23]. These were heated in a furnace for 1 hour at 300 ° C., then heated for 3 hours at 900 ° C at a rate of 3 ° C/min to produce a white powder. This was a result of the calcium carbonate component of the eggshell waste breaking down to yield a calcium oxide powder. [16]. Using the stoichiometric ratio of 1:5, 20g of the snow-white powder and 13.4g of sodium triphosphate were wet milled together in a 250mL bowl containing 100mL deionized water at 500 rpm for 5 hours. Thereafter, this mixture was centrifuged for 30 minutes at a speed of 1000 rpm. It was then dried in an oven for five days at 40 ℃. Thirty stainless steel balls with a diameter of 10mm were used in a planetary ball mill (Retsch® PM 100) during the milling process.

The extraction of nHAp from fish scales was achieved in two batches. In the first batch, the scales were immersed in 1 M hydrochloric acid (HCl) for 24 hours. After 24 hours, they were thoroughly rinsed with reverse osmosis water and soaked in 1 M sodium hydroxide (NaOH) for another 24 hours to remove proteins. The fish scales were rinsed, placed in a beaker with water, and boiled for 20 minutes on a hot plate at 80 ℃. The fish scales were then dried in an oven at 80 ℃ for 2 hours. The scales were placed in crucibles and heated in a furnace at 700 ℃ for 2 hours. In the second batch, the obtained power (FnHAp) was dry ball-milled at 500 rpm for 2 hours using the same milling parameters explain above to obtain (mFnHAp).

2.2 Characterization

2.2.1. Fourier transform infrared spectroscopy analysis

The functional groups that were present in the prepared powders were determined using a Perkin Elmer Universal ATR. A background investigation was initially conducted before scanning. A small quantity of the prepared sample powders was then placed in a sample holder and scanned between 400 and 4500 cm^{-1} with a resolution of 4 cm^{-1}.

2.2.2. X-ray diffraction analysis

To track any potential changes in the crystallinity of the prepared powders, an X-ray diffraction (XRD) analysis was conducted. The patterns were captured using a diffractometer (D8 Advance BRUKER AXS instrument Germany instrument); Cu-Ka radiation (lKa1=1.5406) the patterns were analyzed between 0 and 90 (2 theta). The parameters used were 40 Kv of voltage, 40 mA of current, and 0.5 s of pass time.

2.2.3. High-Resolution Transmission electron microscopic analysis

In a high-resolution transmission electron microscope (HRTEM); (model: Philips CM 120) operating at 120kV, the size, shape, and distribution of the prepared powders were investigated. Before HRTEM observation, tiny amounts of the powders were each dispersed in 10mL of ethanol and sonicated at 10Kv for 10 minutes. Finally, using a Leica microtome (South Africa), thin cross-sections of cryo-microtomed specimens were created and placed on carbon copper grids measuring 100mm by 100mm.

2.2.4 Elemental analysis of samples

The calcium to phosphate ratio of the samples was evaluated using the Energy Dispersive X-ray (EDX) technique. The findings were presented as a percentage weight of all the elements. Due to the interest in estimating the calcium to phosphate ratio that exhibits the component of the tooth's hydroxyapatite, data for carbon, oxygen, phosphorus, and calcium are presented.

2.2.5 pH test

A pH meter (BOECO, BT-675, Germany) with a temperature sensor was used to monitor the pH value. 1.5 g of each of the sample powders (EnHAp, FnHAp, and mFnHAp) was put into a beaker containing 50 mL of deionized water. This mixture was stirred continuously for 10 minutes at a speed of 600 r/min, with pH readings taken every minute.

2.3 Dentin tubule occlusion test

Recently extracted anterior bovine-enamel teeth (15) were acquired at a slaughterhouse in Durban, KwaZulu Natal, South Africa. The bovine teeth were washed thoroughly and sterilised in a solution of 10% chloroxylenol. Using a low-speed water-cooling diamond saw, dentin discs measuring 5 mm by 5 mm by 1 mm were cut below the dentin-enamel junction. The generated dentin disc was then wet-processed for 60 seconds using silicon carbide polishing sheets (600–1000 grits). Before creating the sensitive tooth model, the dentin discs were placed in a resin (AMT composite, South Africa). Afterwards, to open the dentin tubules, the specimens were submerged for five minutes in a citric acid solution with a concentration of 8 wt %. As shown in Table 1, the specimens were randomly allocated into four groups (n = 5) and treated using agitation techniques described by Onwubu, Mhlungu [16].

Table 1: Sample groups

Sample powder	Total
EnHAp	5
FnHAp	5
mFnHAp	5

n=Number of bovine teeth

2.3.1 SEM evaluation of the occluded specimen.

The occluded dentin before and after treatment was evaluated under controlled air conditions using an SEM (field emission, Carl Zeiss) operating at 20 kV. A thin, electrically conductive gold coating was applied to the surface to prevent an electrostatic charge build-up before SEM observation. With the aid of the ImageJ software (National Institute of Health, USA, http://imagej.nih.gov/ij) the ratios of both the occluded and the opened tubules were calculated. The ratios were determined by dividing the area of blocked tubules by the area of all tubules using 10000 magnification photos (n = 5).

2.3.2 Analysis of the data

To study the mean values of the occluded area ratio in the SEM investigation, a 1-way analysis of variance (ANOVA) was conducted using statistical software (IBM SPSS Statistics v28; IBM Corp). Afterwards, a multi-comparison test with Bonferroni correction (=.05) was performed.

3. Results

3.1 Characterisation of the extracted hydroxyapatite samples

The image in Figure 1 (A-C) shows the characteristic absorption peaks of the extracted nHAp powders as revealed by the FTIR analyses. The absorption bands typical of hydroxyapatite characteristics were observed around ~1000 cm^{-1} and ~560 cm^{-1} for all the samples, respectively. This is attributable to the P–O asymmetrical stretching and asymmetric bending vibrations of a $PO_4{}^{3-}$ group [16, 24]. While the carbonate bands at 1450 cm^{-1} were evident in all the samples (Fig. (A-C), the intensity was much more prominent in the EnHAp (Fig. 1C). The presence of the carbonate group in all the samples is attributable to out-of-plane bending mode and asymmetric stretching of $CO_3{}^{2-}$ ions in the structure of the nHAp produced [17]. The reason for the carbonate peak found in all the samples may be associated with the substitution of the $PO_4{}^{3-}$ ions in the structure of nHAp [24, 25]. The hydroxyl group (O-H) bending was observed around ~3750 cm^{-1} but was identified by a small absorption peak for samples (Fig. A and B). In contrast, the absorption band for EnHAp (Fig. 1C) was very visible around 3750 cm^{-1} and it is attributable to the OH stretching [17]. Furthermore, the broadband around ~3450 and 3000 cm^{-1}, and also the sharp band at 1600 cm^{-1} associated with water molecules [16] were evident in EnHAp samples alone (Fig. 1 C). The plausible reason for the absence of water molecules in Figures 1 (A and B) may be connected to the alkaline hydrolysis and mechanochemical processes used in the extraction of the nHAp from the fish scale.

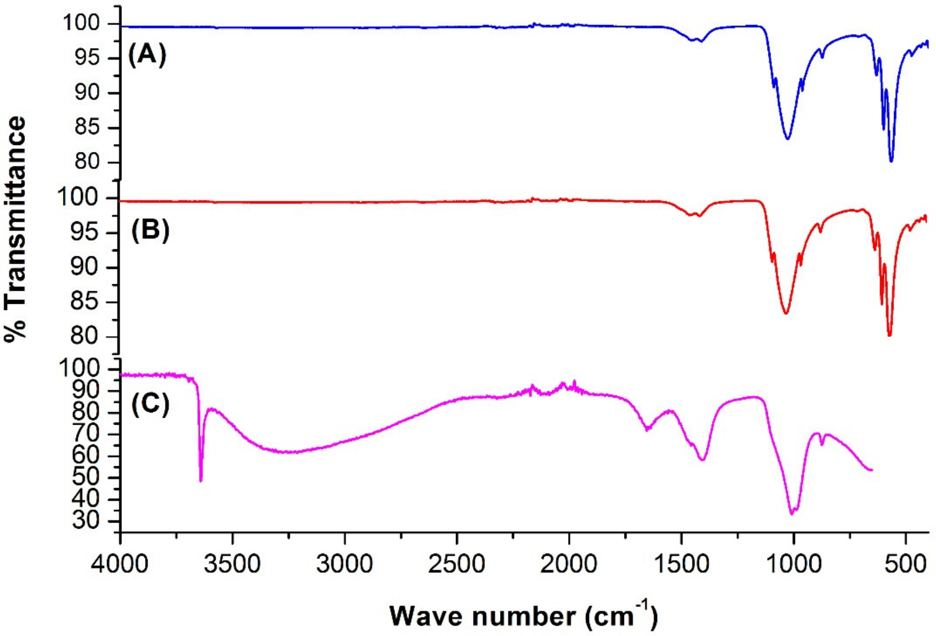

Figure 1: FTIR image of (A) FnHAp; (B) mFnHAp; (C) EnHAp

The image in Figure 2 (A-C) shows the XRD patterns of the extracted nHAp. The pattern of nHAp was observed in all the samples at the diffraction angles marked at 25.8° (002), 31.8° (121), 32.2° (112), 32.9° (030), 39.8° (310), 46.7° (222), and 49.4° (123) are indicative of nHAp, this is also confirmed by the international standard (JCP2-76-0694). In addition, the crystalline peak observed at 29.4° correspond with the calcite while the peak around 34.1° suggests Portlandite (20). Overall, the crystallinity value of EnHAp (82.19%) was higher when compared to FnHAp (67.48%), and mFHAp value (67.22%). The close value in the crystallinity between FnHAp and mFnHAp suggests that milling did not in any way impact the crystallinity of the nHAp extracted from fish scales. The Gaussian plot was used to determine the peak area, and the formula below was used to determine the crystallinity.

Crystallinity Area of crystalline peak Area = of all peaks *100

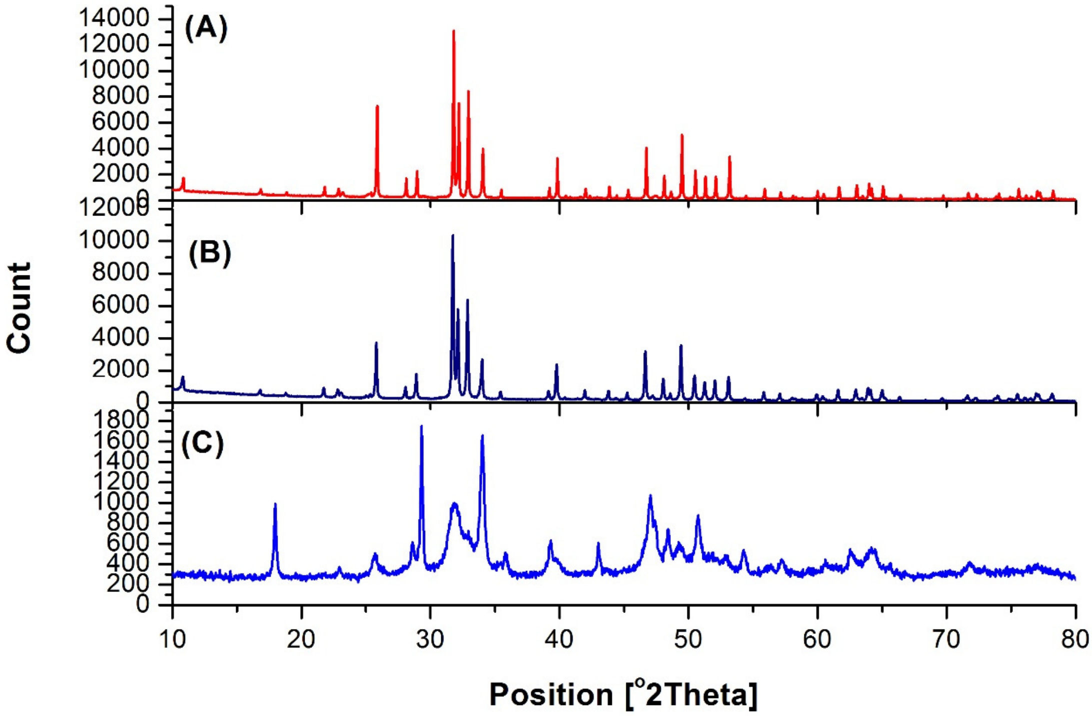

Figure 2: XRD image of (A) FnHAp; (B) mFnHAp; (C) EnHAp

The mineralogical content in the different extracted nHAp (FnHAp, mFnHAp, and EnHAP) obtained from the XRD analysis is further represented in Table 2. The analysis revealed differences in the mineralogical composition of the key minerals identified by the XRD analysis. The highest composition of hydroxyapatite (79%) and portlandite (18%) were measured in mFnHAp, respectively. By contrast, calcite content was higher in FnHAp (12%) compared to mFnHAp (8%), and FnHAp (2.5%).

Table 2. Showing mineral content of the sample powders, as determined by XRD analysis.

Mineral	**Chemical formula**	**FnHAp**	**mFnHAp**	**EnHAp**
Portlandite	Ca $(OH)_2$	3% (Ref. code 96-900-6834)	18% (Ref. code 96-100-1788	13.8% (Ref. code 98-020-2222)

Calcite	$CaCO_3$	12% (Ref. code 96-900-9668	8% (Ref. code 96-900-7688	2.5% (Ref. code 98-016-4935)
Hydroxyapatite	$Ca4(PO4)_6OH_2$	77% (Ref. code 96-230-0274)	79% (Ref. code 96-230-0274	74.8% (Ref. code 96-230-0274)

The International Centre for Diffraction Data (ICDD) reference codes were the reference standard used for XRD analyses. These codes guided the identification of organic and inorganic crystalline materials. FnHAp = fish scale nano-hydroxyapatite; mFnHAp= milled fish scale nano-hydroxyapatite; EnHAp= eggshell nano-hydroxyapatite; XRD = X-ray diffraction

The outcomes of the EDX analysis for the sample groups are shown in Table 3. The elements carbon (C), calcium (Ca), oxygen (O), phosphorus (P), and sodium (Na) were present in both the EnHAp and the mFnHAp. The presence of sodium (Na) was the visible absence in FnHAp. The analytical results suggest that all sample groups were rich in calcium and phosphorus in the form of carbonate and phosphate. The relative ratio of calcium and phosphorus (Ca/P) was found to be different for each sample. The mFnHAp had an approximate value of 1.9, which is slightly higher than the 1.67 ratios found in human bones and teeth. By contrast, the Ca/P ratio for FnHAp and EnHAp were slightly off the mark of the stoichiometric ratio of 1.67 ratios. The feasible theory might be connected to the fact that natural nHAp is non-stoichiometric since it contains trace elements like sodium and magnesium [26, 27].

Table 3: Elemental analysis of the samples

Element (Weight in %)	Sample groupsSample groups		
	EnHAp	FnHAp	mFnHAp
Carbon (C)	17.5%	10.5%	15.3%
Calcium (Ca)	26.9%	66.3%	21.4%
Oxygen (O)	39%	15.6%	45%
Phosphorus (P)	13%	7.9%	11.1%
Sodium (Na)	3.0%	0%	4.8%
Magnesium	0.6%	0%	2.4%
Ca/P	2.1	2.1	1.9

The HRTEM image in Figure 3 (A-C) shows the surface morphology of the sample powders. There were visible differences in the surface morphology of the nHAp extracted from eggshells and fish scales. The image in Figures 3A and B shows that the nano-hydroxyapatite from fish scales (FnHAp and mFnHAp) particles presented an irregular spherical morphology. After milling at 500 rpm for 2 hours, the mFnHAP (Fig 3B) showed an increase in surface smoothness in comparison with FnHAp (Fig 3A). By contrast, an irregular-rod-like structure was observed for the eggshell nano-hydroxyapatite (EnHAp). The particles also appeared to assemble into short chains that stuck together into clusters (Figure 3C). In addition, the particle size of the sample powder measured using Image J software showed that the EnHAp had a mean length of 11.6±2.7nm. By contrast, the mean length measured for FnHAp and mFnHAp were 190±81.8 and 74.6±33.7, respectively.

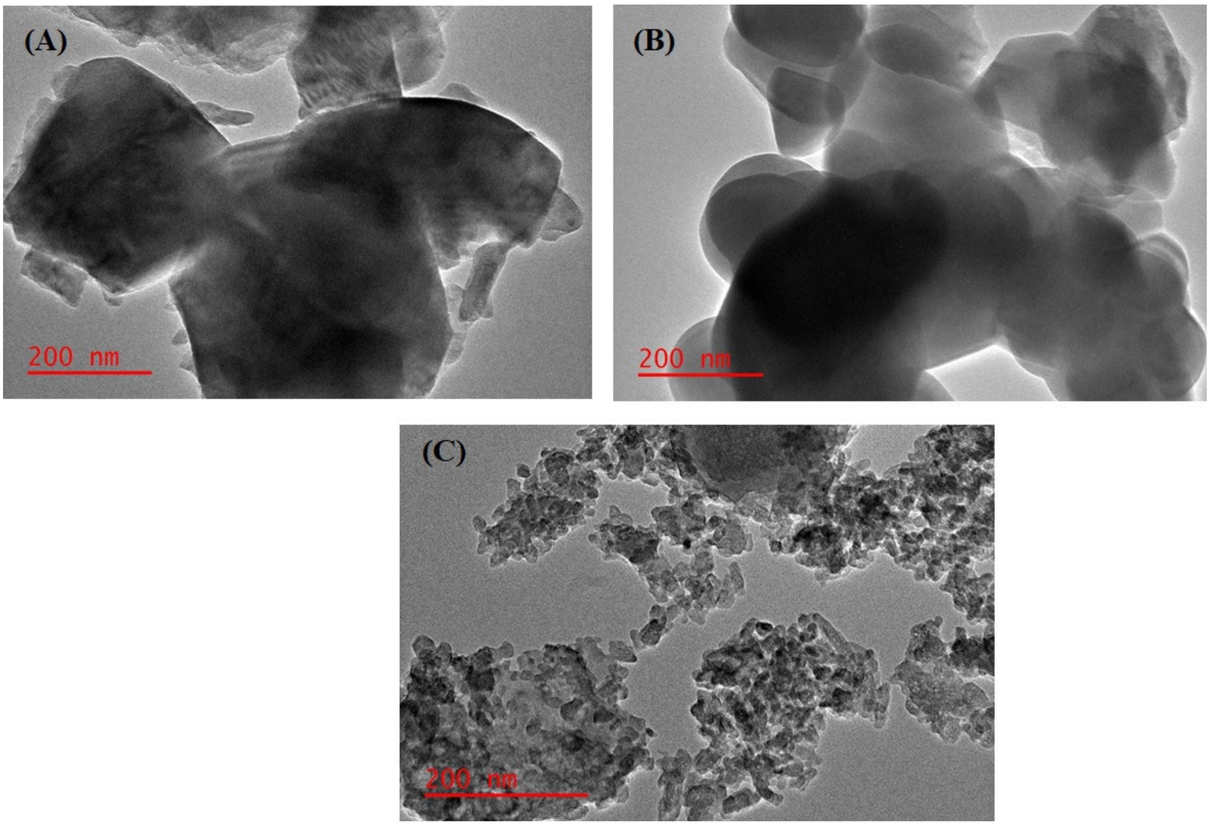

Figure 3: HRTEM micrograph of (A) FnHAp; (B) mFnHAp; (C) EnHAp

Figure 4 shows the pH of the sample powders in deionised water and citric acid solution with initial pH of 2.06. The findings indicated that EnHAp had the highest mean pH value in deionized water (13.09), whereas the mFnHAp had the lowest value (10.77). All the sample powder had alkaline characteristics that may be attributable to the elevated calcium content. EnHAp had the best buffering characteristics when compared to both FnHAp and mFnHAp from fish scales in the sample powder study using the citric acid solution at pH 2.06 since it effectively neutralized the pH of the citric acids (Fig 4).

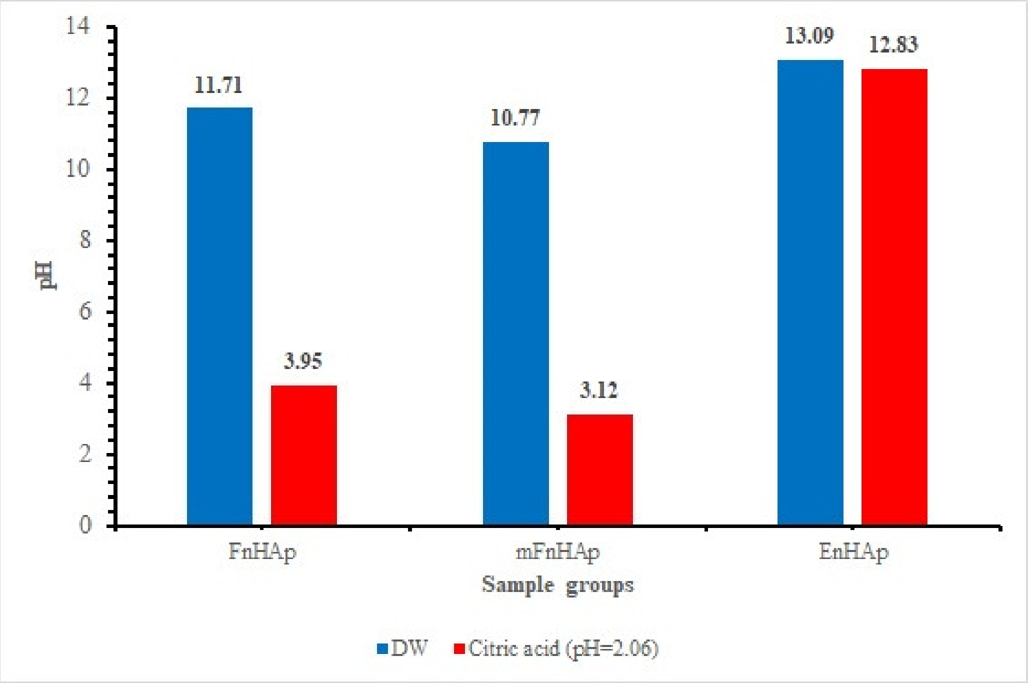

Figure 4: Buffering characteristics of samples

3.2 Dentin tubule occluding characteristics

FESEM was used to examine the morphological alterations of the occlusion of the dentin tubule before and after treatment (agitation). The samples treated with EnHAp (Fig 5(C2)) and mFnHAp (Fig 5(B2)) had more occluded areas than the samples treated with FnHAp (Figure 5(A2)). All samples show visible evidence of dentin tubule occlusion after agitation. The nanoparticles can be seen on the surface of the dentin specimens.

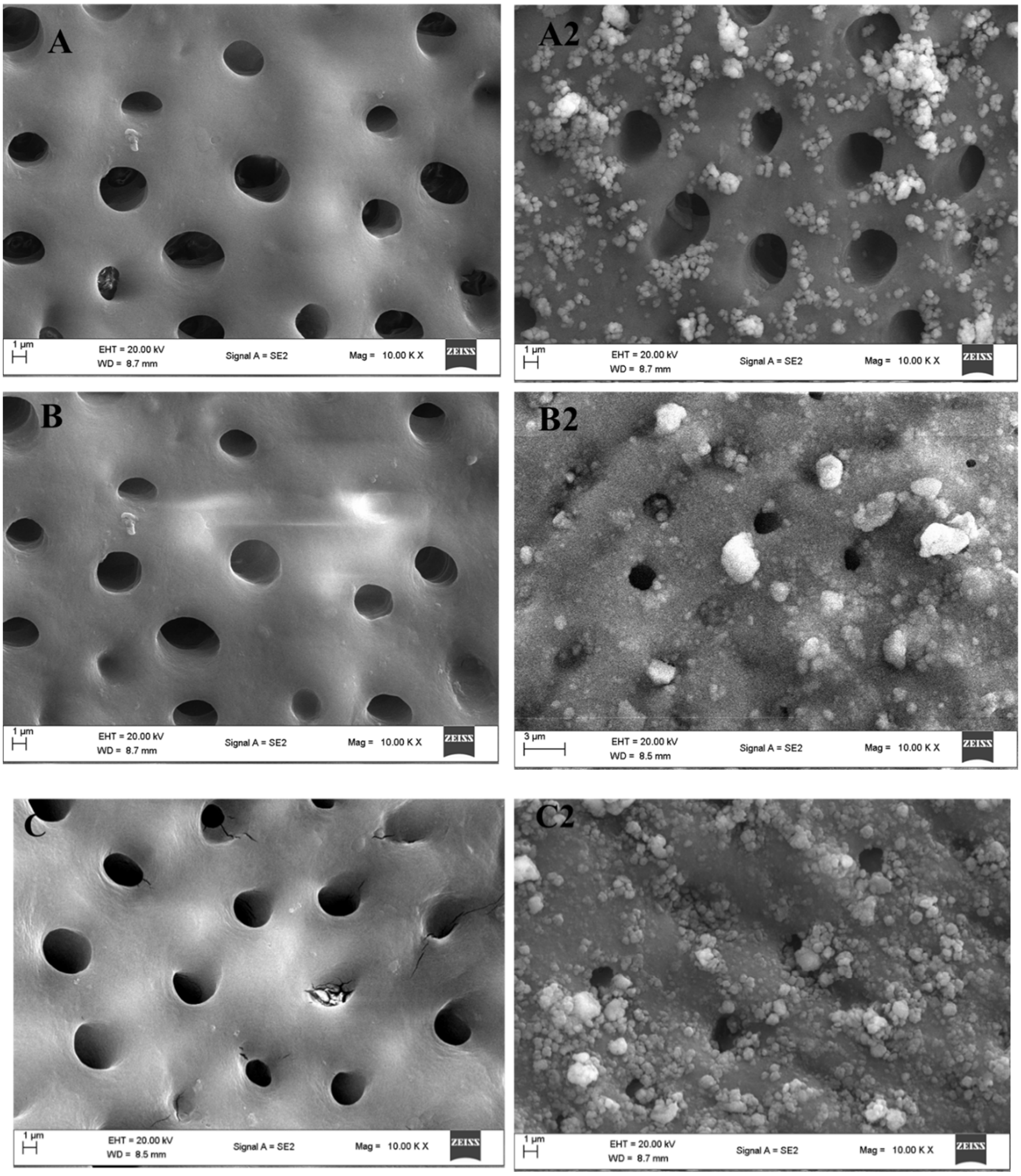

Figure 5: Remineralization test (A=FnHAp; B=mFnHAp; C=EnHAp (1 represents after agitation)

The mean % occluded area of the dentin tubules for the treated specimens is given in Table 4. The ANOVA value shows that the total mean % ratio of the dentin tubules occluded area for the specimens treated with sample powders (EnHAp, FnHAp and mFnHAp) was statistically significant ($p<0.001$). The results indicate that EnHAp had the highest % mean occluded area (78.2 ± 1.5%) while the lowest was measured for the FnHAp group (59.7 ± 1.7%). According to the results of the Bonferroni test, the percentage of tubules occluded for the EnHAp group was statistically higher than that of the FnHAp group and the mFnHAp group (P=0.002 and p0.001, respectively). In comparison to the FnHAp group, the % occluded area measured for the mFnHAp group was higher (p 0.001).

Table 4 ANOVA test comparison of the occluded area

Treatment group	***N***	**Mean ± SD**	**Standard error**	**95% confidence interval**		***p*-Value**	**Bonferroni's correction**
				Lower bound	**Upper bound**		***p*-Value**
EnHAp	5	78.2 ± 1.5	0.680	76.332	80.108	<0.001	$0.002^{1,3}$
FnHAp	5	59.7 ± 1.7	0.753	57.629	61.811		$0.001^{1,2}$
mFnHAp	5	72.9 ± 2.1	0.939	70.332	75.548		$0.001^{3,2}$

Abbreviation: SD, standard deviation. Note: the superscript numbers indicate the mean differences between sample groups. EnHAp= Eggshell nanohydroxyapatite, FnHAp= Fish scale nanohydroxyapatite, mFnHAp= milled fish scale nanohydroxyapatite.

4. Discussion

One of the most common concerns in dentistry is dentin hypersensitivity (DH). This clinical condition has the potential to impede and restrict people during daily activities, which harms the person's quality of life [28]. According to Balhuc, Campian [29], the use of more complex products and combinations between nano-hydroxyapatite (nHAp) and various compounds is an innovative method of significantly reducing DH and remineralizing dentinal tubules. While synthetic nHAp is commonly used in biomaterial applications, the need for environmental sustainability products has created a niche for bio-waste materials. In this present study, we comparatively assessed the remineralisation characteristics of nHAp extracted from waste eggshells and fish scales. The results showed that there were slight variations in the physicochemical characteristics of the nHAp extracted (Figure 1). While the nHAp from eggshells had higher crystallinity, the mineralogical content of hydroxyapatite detected was, however, higher in the fish scale nHAp (Table 2).

The surface morphology also revealed variations between the nHAp extracted from eggshells and that of fish scales (Figure 3). While the study results showed that milling of the nHAp extracted from fish scale does not negatively impact the physicochemical characteristics, the mFnHAp, however, showed smother surface morphology and reduced particle size after milling (Figure 3). This may be attributable to the effect of the mechanical energy during milling - which could have triggered the transition of the aggregated FnHAp to a more smother mFnHAp (Figure 3). Another study reported that milling stimulates transitions of substances in all aggregation states due to the impact of mechanical energy [30]. Based on the study results, it is reasonable to presume that the physicochemical characteristics of the nHAp such as the crystallinity, particle size and surface morphology vary because of the source of bio-waste, method of extraction, pH and temperatures [31].

Furthermore, the results indicate that the buffering characteristics of EnHAp against citric acids were superior to those measured for nHAp extracted from fish scales (Figure 4). This shows that EnHAp will offer better protection against enamel demineralisation from acidic substances, as it could neutralize citric acid effectively. The implication of this to oral health is that EnHAp could offer high bioavailable calcium that is crucial for tooth remineralisation [17, 32]. This is supported by the FESEM image in Figure 5 which shows that EnHAp had the most increase in dentin tubules occlusion. Agreeing with Tschoppe, Zandim [33], it thus means that the higher pH values of the nHAp slurries favour tooth remineralisation. More so, statistical results indicate that there was a statistically significant difference in the treated dentin specimens ($p<0.001$). The formulated study hypothesis was rejected based on the obtained results.

Overall, the EnHAp group had the highest mean % occluded area of the dentin tubules (Table 4). The plausible difference in the dentin tubules measured may be associated with the physicochemical characteristics of the nHAp extracted. This aligns with Kong, Xiao [34] that the performance of nHAp is influenced by its crystallinity, particle size, and surface morphology. It thus means that the smaller particle size of EnHAp and the higher pH values favour its remineralisation characteristics over the fish scale nHAp. Besides, the particle sizes measured for the nHAp extracted are smaller than the size of tubules close to the Dentin-enamel junction, which is about 3-5 µm [35]. This meant that smaller particles of nHAp could easily be attached to dentinal surfaces and eventually occlude exposed dentin tubules. This could explain the dentin tubule occlusion in all the tested nHAp from both fish scales and eggshells (Figure 5).

Another plausible reason may be attributable to polarity and the large surface proportion of the atomic number of nHAp, which could allow the particles to bind to collagen and hydroxyapatite from dentin [36].

5. Conclusion

In summary, it is reasonable to conclude that nHAp from natural bio-waste such as eggshells and fish scales represent a revolutionary material that is useful in the remineralization of dentin tubules. The physicochemical characteristics of the extracted nHAp suggest that they provide a better source of free calcium (Ca) and phosphorus (P), which are key elements in the process of dentin tubule remineralization. Overall, the study, find slight variations in the physicochemical characteristics of the nHAp extracted from fish scales and eggshells. It was established that eggshell nHAp (EnHAp) showed superior dentin tubule occluding characteristics than those of fish scales (FnHAp and mFnHAp). Understanding all the mechanisms by which these particles interact with the intraoral environment is crucial given the growing significance of nHAp biomaterial applications. This study thus provides a benchmark for further advanced studies on utilising natural nHAp in dentistry.

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Conflict of interest

None declared

Data availability statement

On reasonable request, the corresponding author will provide the datasets generated during and/or analysed during the current study.

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CHAPTER FOUR – CRITICAL DISCUSSION

The use of bio-based materials such as hydroxyapatite has gained popularity in recent years. The unique properties of these materials such as their non-toxicity, abundant availability, biocompatibility, biodegradability, and physical, chemical, and structural properties have led to an increase in interest among scholars for their use in different applications (Battegazzore *et al.* 2019). In dentistry, the use of hydroxyapatite particles, particularly in the nano-size has emerged as a promising material for remineralising dentin. This is highly relevant given that non-carious damage to the tooth structure integrity from erosion, attrition, abfraction, and abrasion significantly contributes to the prevalence of dental problems such as dentin hypersensitivity (Arnold, Prange and Naumova 2015).

While this study acknowledges the fact that there is no gold standard material to effectively remineralised tooth damage (Schmidlin and Sahrmann 2013; de Melo Alencar *et al.* 2019), the use of hydroxyapatite from biowaste in remineralising dentine will add value towards waste management. Equally important, nHAp is a natural component of the human tooth and bones making up about ~90% of the enamel and ~70% of the dentin (Neel *et al.* 2016), which thus make nanohydroxyapatite ideal for remineralisation of damaged dentin tubules. It has also been shown that modifying nHAp with mesoporous silica improves the latter acid resistance which is vital in the control of non-carious erosion (Yu *et al.* 2016). Hence, this study's aim was to synthesize a mesoporous nanohydroxyapatite bionanocomposite from natural waste by the application of a mechanochemical method for potential use for dentistry as a remineralizing agent. The hypotheses tested in this study were:

Hypothesis one:

H_0: There is no significant difference in the occluding abilities of the nHAp extracted from eggshells (EnHAp) and those extracted from fish scales (FnHAp).
H_1: There are significant differences in the occluding abilities of the nHAp extracted from eggshells and fish scales.

Hypothesis two:

H_0: Modification of EnHAp with mesoporous silica (MSN@nHAp) had no difference in its protective effect against erosive acids.

H_1: Modifying EnHAp with mesoporous silica (MSN@nHAp) significantly improved its protection against erosive acids.

4.1 Research hypothesis one: Comparing the occluding abilities of the nHAp extracted from eggshells and fish scales

Dentin hypersensitivity is a common oral disease that affects nearly 43% of the adult population worldwide (Saeki *et al.* 2016; Onwubu *et al.* 2019c), and thus poses a challenge for an oral care provider to effectively manage. Recent studies which are supported by clinical evidence reported that nano-hydroxyapatite (nHAp) based desensitising agents showed promising clinical improvements in the treatment of DH (Suresh *et al.* 2018; Vano *et al.* 2018). However, most nHAp uses are synthetic. Given the concerns regarding the management and usage of bio-waste, there have been numerous interests in finding value in converting biowaste into value-added products. Considering this, it became pertinent to extract nHAp from bio-waste. Paper 1 investigated the remineralization and acid resistant characteristics of nanohydroxyapatite produced from eggshell waste via mechanochemistry. According to West, Seong and Davies (2015), enamel erosion is one of the main predareposing factors that contribute to the onset of DH. It thus meant that developing materials that is resistance to erosive attacks will be critical in the prevention to enamel erosion.

The nHAp from eggshells was prepared using a mechanochemical approach. This technique is reported to be gaining increasing attention among researchers due to its simplicity, low cost, reproducibility and environmental friendliness (Baláž 2018). The milling condition of 20g of calcined eggshell powder and 13.4g of sodium triphosphate milled at a speed of 500 rpm for 5 hours in a 250mL bowl containing 100mL water (Paper 1). The success of the nHAp extraction was confirmed using different traditional characterisation techniques such as Field Scanning Electron Microscope (FESEM), X-ray Diffraction (XRD), Fourier Transform Infrared (FTIR), and High Transmission Electron Microscope (HRTEM).

Paper 1 established that nHAp was successfully produced from eggshell waste after 5 hr of milling. It was found that the produced nHAp (EnHAp) was effective in neutralizing common dietary acids. This is highly important when one considers that dietary acids negatively affect the structural integrity of the tooth enamel (Jain *et al.* 2012). This is also supported by the HRTEM results which show that the dietary acids used in the study had minimal effect on the enamel surface integrity (Paper 1, Figure 6(c)). Also, and of interest to dentistry, the nHAp showed complete occlusion of the dentin tubules. This is vital in the management of DH. The finding aligns with the notion that blocking the open dentin tubules will invariably control the hydrodynamic process (Sykes 2007; Onwubu *et al.* 2019a).

Apart from eggshells, fish scales are another bio-waste that is rich in natural hydroxyapatite. Manuscript 1 was based on the comparative assessment of the remineralization characteristics of Nano-hydroxyapatite extracted from fish scales and eggshells. The nHAp from fish scales was prepared using direct calcination after alkaline hydrolysis (FnHAp) followed by the mechanochemical method (mFnHAp) while the eggshell nHAp (EnHAp) was produced as detailed in Paper 1.

Manuscript 1 sought to answer the following research questions which are:

- Will there be any differences in the physicochemical characteristics of the nHAp extracted from fish scales and eggshells?
- Will ball-milling the extracted fish scales after direct calcination make any differences in the physicochemical characteristics?
- Will there be any difference in the occluding characteristics of the nHAp based on the source and method of processing?

Manuscript 1 established that nHAp extracted from eggshells (EnHAp) showed superior dentin tubule occluding characteristics than those of fish scales (FnHAp and mFnHAp). It was found that there were slight variations in physicochemical characteristics such as the Ca/P ratio, crystallinity, particle sizes and surface morphology of the nHAp extracted (Manuscript 1). The study confirmed that ball-milling of FnHAp had an impact on the surface morphology of the final nHAp produced. Based on the study finding, the tested hypothesis was accepted as there were significant differences in the occluding abilities of the nHAp extracted from eggshells and fish scales. Manuscript 1 thus provides a benchmark for further advanced studies on utilising natural nHAp in dentistry.

4.2 Research hypothesis one: Modification of nHAp with mesoporous silica improves acid resistance characteristics

It has been suggested in the literature that the novel approach to the treatment and management of DH is the use of various nanomaterials (Onwubu, Mdluli and Singh 2019). Mesoporous silica (MSN) and nanohydroxyapatite (nHAp) had gained interest among scholars due to their antibacterial action, and physical, mechanical, and biological characteristics (Yu *et al.* 2016). Although MSN@nHAp composites have been previously reported in the literature with promising acid resistance results (Yu *et al.* 2016), they are, however, synthesized through the precipitation process which involves the use of toxic chemicals, and it is time-consuming (Zhou and Lee 2011; Googerdchian *et al.* 2018). Given this concern, Paper II reports on the invitro assessment of the acid resistance characteristics of mesoporous silica/nanohydroxyapatite (MSN@nHAp) biocomposite synthesised through the mechanochemical method. The modification was achieved by dry milling 1g of the mesoporous silica with 10g of nHAp (EnHAp).

It was found that the composite exhibits both crystalline and amorphous structure which is evidence of surface modification of nHAp (crystalline) with mesoporous silica (amorphous). It was demonstrated in Paper II that the MSN@nHAp exhibits superior acid resistance characteristics (Figure 3). It was assumed that the superior acid resistance properties observed for MSN@nHAp confirmed that the mechanochemical technique can be a useful tool in the synthesis and surface modification of valuable biomaterials (Paper II). Based on the study finding, the tested hypothesis was accepted as modifying EnHAp with mesoporous silica significantly improved the material protection against erosive acids.

4.3 Conclusion

The critical discussion aims to connect and show the linkage between the research objectives, study hypothesis and published articles. The experimental findings have provided evidence that nHAp can be extracted from bio-waste such as eggshells and fish scales. This is incredibly important from a South African perspective as it will help towards a cleaner and greener environment. The findings also provided evidence that nHAp from bio-waste such as eggshells and fish scales represent a revolutionary material that is useful in the remineralization of DH. 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 FIVE – GENERAL DISCUSSION

Oral diseases such as tooth sensitivity are a public health concern that affects the well-being of many people suffering from the pain and discomfort associated with it. The use of bio-based materials in their nano-size forms has brought about tremendous growth and advantage in dental practice, particularly in the management of tooth sensitivity. While there is yet to be an established gold standard technique for the permanent treatment of DH, the use of nanohydroxyapatite is a gain changer in the treatment of DH due to its similarities to bones and teeth. The focus of this study was to synthesize a mesoporous nanohydroxyapatite bionanocomposite from natural waste by the application of a mechanochemical method for potential use in dentistry as a remineralizing agent. The study, therefore, adopts a quantitative research approach and an experimental research design in assessing the remineralization and acid resistance characteristics of the material extracted from both eggshells and fish scale waste. This chapter concludes by drawing on the discussion of the research objectives to provide recommendations for the study

5.1 REVISITING THE RESEARCH OBJECTIVES

The research objective was achieved in three phases.

Objective One: Preparation and extraction of nHAp from bio-waste.

The sub-objectives are to:

1. Extract nHAp from eggshells using calcination and mechanochemical method.
2. Synthesize mesoporous nanohydroxyapatite bionanocomposite (MSN@nHAp) from eggshells using the mechanochemical method.
3. Extract nHAp from fish scales using alkaline hydrolysis and mechanochemical method
4. Compare the physicochemical characteristics of eggshells and fish scales nHAp using different analytical techniques.

The findings of this study confirmed that nHAp was successfully extracted from bio-waste materials such as eggshells and fish scales via the mechanochemical method (Paper I and Manuscript 1). It was found that the ball-milling process causes changes in the surface morphology of the nHAp extracted from fish scales (Manuscript 1). The study confirmed that there were slight variations in the physicochemical characteristics of the nHap extracted from fish scales and eggshells (Manuscript 1). There were variations in the Ca/P ratio, particle size, surface morphology, and crystallinity (Manuscript 1). Equally, the findings show that mesoporous silica was successfully used to modify nHAp via the mechanochemical method.

Objective Two: In vitro assessment of the cytotoxicity of nHAp extracted using mouse fibroblast cell culture.

In line with achieving the research objective which is to assess the cytotoxicity of nHAp extracted using fibroblast cell culture, the finding of the cytotoxicity assay show that the material had low toxicity (Paper 1).

Objective Three: Assessing in vitro the fitness of purpose of the bio-based nHAp

The sub-objectives are to:

1. Assessed the remineralisation characteristics of nHAp extracted from eggshells.
2. Comparatively assessed the remineralisation characteristics of nHAp extracted from waste eggshells and fish scales.
3. Assessment of the acid resistance characteristics of mesoporous silica/nanohydroxyapatite (MSN@nHAp) in comparison with nHAp from eggshells.

This study conclusively showed that the EnHAp exhibits outstanding remineralization and occluding properties (Paper 1). Furthermore, the occluding properties of EnHAp were superior to those measured for FnHAp and mFnHAp (Manuscript 1). Equally, MSN@nHAp exhibits superior acid resistance in comparison with nHAp alone (Paper II). This further confirmed that modifying nHAp with silica improved the acid resistance properties of the biomaterial.

5.2 LIMITATION

While the nHAp extracted from eggshells and fish scales waste was promising, it is, however, limited to in vitro assessment of the remineralization characteristics. Further studies will seek to assess clinically the effectiveness of nHAp extracted from bio-waste in the treatment and remineralization of damaged teeth.

5.3 RECOMMENDATIONS

Considering the study findings and the research objectives, the researcher proposes the following recommendations:

5.3.1 Clinical evaluation of nHAp

To fully assess the effectiveness of nHAp extracted from bio-waste in remineralizing damaged teeth, a clinical evaluation of the material will be important. This will pave the way for its potential commercialisation in the use of toothpaste formulation.

5.3.2 Using bio-waste as a tool for environmental management

The data provided by the Food and Agricultural Organisation (FAO) indicates that in the upcoming years, global eggshell waste production is likely to hit 86.8 million tons. This trend has increased given that the world population has just hit over 8 billion people. It thus meant that waste management will become problematic in the future. The development of valuable sustainable products from eggshell and fish waste would thus serve as a double-purpose choice not only to gain a sustainable source of material for nHAp extraction but also vital in the environmental problem of waste management. This will also help towards achieving a circular economy, which is high towards a cleaner and greener environment. More importantly, the use of bio-waste such as eggshells and fish scales in producing value-added products will go a long way in meeting the United Nations Sustainable Development Goal (SDG13) that speaks on urgent action to combat climate change and its impacts on the environment.

5.3.3 Valuable materials for oral healthcare management

The core and vital ingredients in toothpaste formulation are abrasive materials. This study highly recommends the use of nHAp extracted from eggshells and fish scales in toothpaste formulation. This will help produce valuable biomaterial for the treatment of tooth sensitivity. Besides, the anti-acid properties support its use in toothpaste formulation for oral healthcare management.

5.3.4 Mechanochemical method

The salient aspect of this study suggests that mechanochemistry is a useful technique in the synthesis and surface modification of valuable biomaterials. This study, therefore, recommends the technique for the extraction, modification, and synthesis of value bio-based materials. This is attributed to the fact that the technique is environmentally friendly, simple to use, cheaper and requires less time and energy to produce valuable products.

5.4 CONCLUSION

The aim of the study, namely, to synthesize a mesoporous nanohydroxyapatite bionanocomposite from natural waste by the application of a mechanochemical method for potential use for dentistry as a remineralizing agent was achieved as illustrated by the study findings. The study hypotheses were extensively elaborated in the published and developed manuscripts. The study provides a benchmark for the use of nHAp extracted from bio-waste in dental applications. The study further provides new evidence and an approach to the preparation of valuable biomaterials through the modification of MSN@nHAp bionanocomposite using the mechanochemical method.

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ADDENDUM: CONVENTIONAL BRUSHING TEST

The morphological changes of the dentin tubule occlusion before and after treatment conventional brushing were studied by FESEM. The samples treated with EnHAp (Fig 1(C2)) and mFnHAp (Fig 1(B2)) had more occluded areas than the samples treated with FnHAp (Figure 1(A2)). All samples show visible evidence of dentin tubule occlusion after brushing for 2 min.

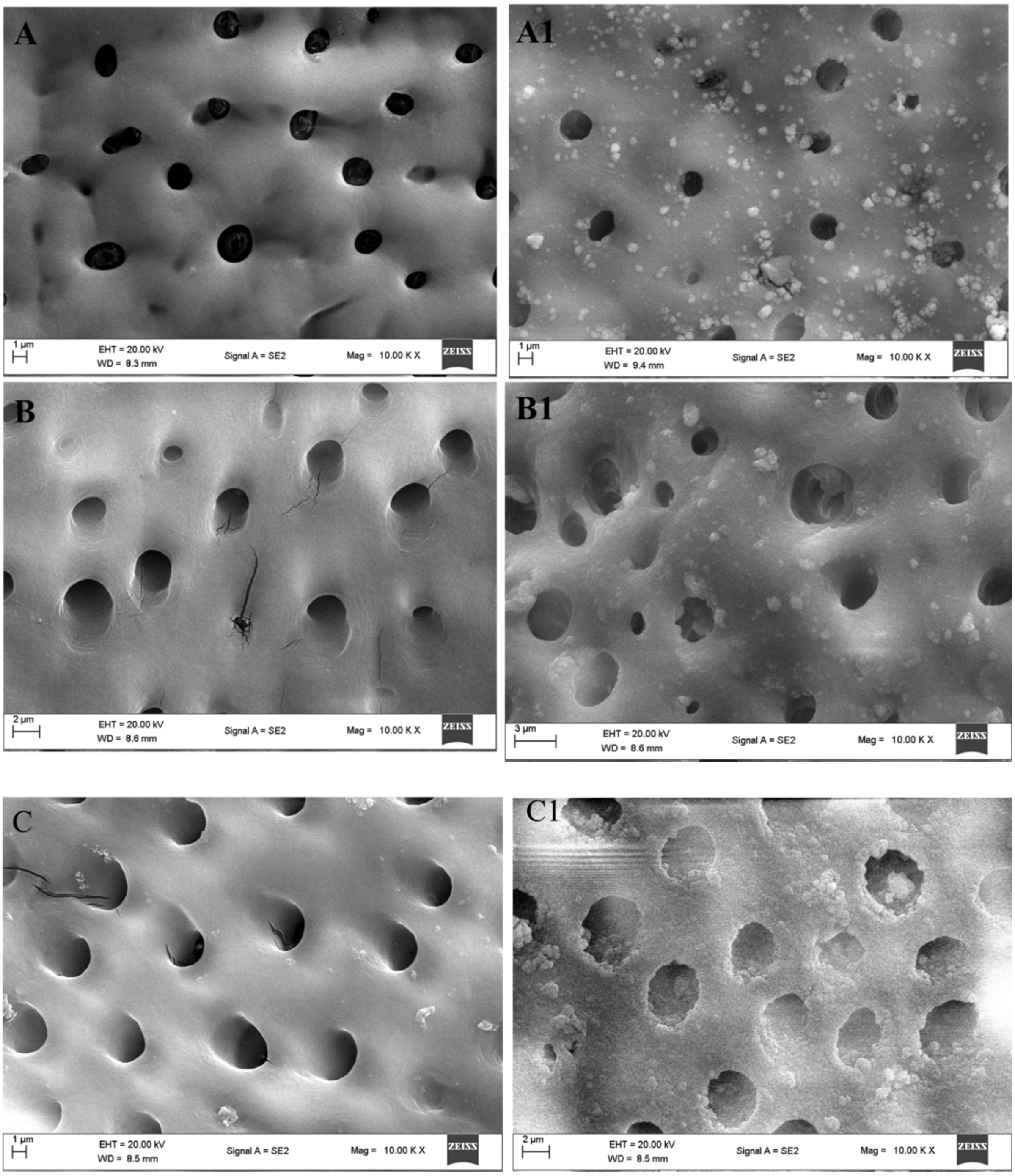

Figure 1: Brushing test (A=FnHAp; B=mFnHAp; C=EnHAp (1 represents after brushing)