Original Article

Fabrication & Analysis of the Nanohydroxyapatite / Chitosan Membranes for Rejuvenation of Periodontal Tissues

Nausheen Ashraf[1], Maryam Saeedullah[1] and Bakhtawar Yaqoob[2]

Evaluate the Structural & Antibacterial Properties for Periodontal Tissue Regeneration

ABSTRACT

Objective: To fabricate Nanohydroxyapatite/Chitosan barrier membranes loaded with Amoxicillin nanoparticles and evaluate their structural and antibacterial properties for periodontal tissue regeneration

Study Design: It is a cross sectional study.

Place and Duration of Study: This study was conducted at the RIPS (Riphah Institute of Pharmaceutical Sciences) Islamabad in collaboration with IST (Institute of Space and Technology) Islamabad April 2020 to February 2021 for a duration of ten months.

Materials and Methods: The membranes of nanohydroxyapatite/chitosan incorporated with the drug loaded nanoparticles were fabricated by solvent casting method followed by lyophilization. The membranes were characterized using SEM. Antibacterial activity of the membranes was observed using disc diffusion method and the zone of inhibition was measured against Staphylococcus aureus and Escherichia coli

Results: The membranes exhibited a porous structure with all the components blended well. The zone of inhibition measured against Staphylococcus aureus was 24.6±0.4 mm and that against Escherichia coli measured 22.3±0.4mm.

Conclusion: It can be suggested from the results that the fabricated membranes incorporated with drug loaded nanoparticles show their potential to be used in the periodontal regenerative therapy possessing adequate structural and antibacterial features.

Key Words: Nanohydroxyapatite membranes, Antibacterial property, Regenerative therapy, Lyophilization

Citation of article: Ashraf N, Saeedullah M, Yaqoob B. Fabrication & Analysis of the Nanohydroxyapatite / Chitosan Membranes for Rejuvenation of Periodontal Tissues. Med Forum 2023;34(2):2-6.

INTRODUCTION

One of the leading causes of tooth loss is periodontitis. It is an inflammatory disease, involving the supporting tissues of teeth leading to the impairment of the periodontium[(1)]. Worldwide the percentage of the adult population having moderate form of periodontitis is around 35% while 15% are affected with a severe form [(2)]. Research on periodontal disease is limited in Pakistan, however according to the national oral health survey and some studies giving details on the status of periodontal disease, report its prevalence up to 98% [(3)]. Regeneration of the periodontium is the utmost objective of the periodontal therapy. The use of membranes for guided tissue regeneration is the most approved treatment[(4)].

[1.] Department of Dental Materials, HITEC-IMS Dental College, Taxila.
[2.] Department of Dental Materials, Islamic International Dental College, Islamabad.

Correspondence: Dr. Nausheen Ashraf, HITEC-IMS Dental College, Taxila.
Contact No: 0321 3889865
Email: nausheen.ashrf@gmail.com

Received: July, 2022
Accepted: December, 2022
Printed: February, 2023

The migration of gingival epithelium is prevented by these barrier membranes which allow the periodontal ligament and bone tissue to regenerate during the healing phase[(5)]. Many nonabsorbable and absorbable membranes have been developed and studied in this regard but these conventional membranes possess inadequate mechanical, structural, bio-functional, antibacterial and drug releasing properties [(6)]. For example, the nonabsorbable membranes require the removal by a second surgical process which causes re-infection, pain, irritation and financial hardships whereas the degradable lack adequate stability and firmness required at the minimum for 30 to 40 days. Ideal membranes for periodontal generation should display appropriate bio-compatibility, bio-degradability, antibacterial, mechanical and physical properties [(6)]. Thus, the optimization of the membranes is the need of the hour.

A new conception of periodontal regeneration is achieved through tissue engineering. It is an emerging field of science which aspires to resemble natural tissue by using structures with or without the addition of cells to reestablish the function of the impaired tissues. Currently nanotechnology plays a pivotal role in this regard which is an important strategy towards long term treatment and advancement in the periodontal regeneration therapy.

Bio ceramics have been used and studied extensively in both bone tissue engineering and in the regeneration of

the periodontium [7]. Nanohydroxyapatite (nHA) a bio ceramic stands out to be the most promising material for use in biomedical applications [8].This is because it possess a high surface area with ultrafine composition and its resemblance to the natural constituents of the bone. Various studies have reported that nanohydroxyapatite has the ability to increase the protein synthesis of periodontal ligament cells which improves the alkaline phosphatase activity leading to cell differentiation and promoting regeneration of the periodontal tissue[9]. Despite being bioactive, bio ceramics are brittle with a low mechanical strength and are difficult to shape[10]. However, these limitations can be overcome by combining them with the polymers either natural or synthetic.

This blend of a bio ceramic with the polymer is a recent strategy to simulate the composition of the osseous matter. These hybrid or composites representing the amalgamation of two or more constituents each having its own advantages and unique features results in the overall enhancement of the whole system[11], thus achieving the desired results. Most of these available composites are based on the use of synthetic polymers like PCL (polycaprolactone), PLGA (polyglycolic acid) and PLA (polylactic acid) which have adequate strength but lack bioactivity[12]. The ones based on collagen or its derivatives have reported favorable results and better osteoconductivity but the high cost of collagen, high degradation rate, its extraction from the animal tissue is not only related with a risk of transmission of disease but has also given rise to ethical and religious issues which have limited its clinical usage[13]. Alternatively, a polymer which has gained much attention in the biomedical field is Chitosan. It is an attractive material which is known for its excellent biocompatibility, biodegradation, antimicrobial, wound healing ability, nontoxicity and cost effectiveness. It is proposed that combining nHA with chitosan is ideal for various biomedical applications.

Hence there is a considerable need for the local production of modified membranes in Pakistan which are not only cost effective, easily available but are also acceptable clinically, religiously and ethically. Therefore, in the current study chitosan and nanohydroxyapatite with drug loaded nanoparticles were chosen to develop membranes that have adequate structure and possess antibacterial properties.

MATERIALS AND METHODS

Materials: List of Chemicals Used in Study

S.No	chemicals	Manufacturer	Cas.no
1.	Acetic Acid	Sigma-Aldrich	64-19-7
2.	Chitosan (LMW)	Sigma-Aldrich	7012-76-4
3.	1-ethyl-3(3dimethyl aminopropyl) Carbodiimide.(EDC)	Sigma-Aldrich	22572-40-3
4.	N-Hydrosuccinimide (NHS)	Sigma-Aldrich	6066-82-6
5.	Sodium Hydroxide.(NaOH)	Sigma-Aldrich	1310-73-2
6.	De-ionized water	Sigma-Aldrich	7732-18-5
7.	Nanohydroxyapatite powder(nHA)	AMTECH	-

Nanohydroxyapatite was kindly gifted by AMTECH (Advanced Materials Technologies) Islamabad who fabricated and characterized it[14].

Preparation of Nanohydroxyapatite Membranes: The membranes were fabricated using solution casting method followed by lyophilization[15]. The amount of Chitosan and Nanohydroxyapatite(nHA) were taken in the ratio of 60:40. Chitosan 4 gm was dissolved in 2% aqueous acetic acid (50ml) and the solution was stirred until the entire polymer was dissolved on the magnetic stirrer. Chitosan nanoparticles of Amoxicillin (ACNs) (equivalent to 100mg) were added to this Chitosan solution and stirred until dissolved.

The Nanohydroxyapatite powder (3gm) was dissolved separately in deionized water(50ml) under continuous stirring. EDC/NHS in the ratio of (3:1) used as a cross linker were added to the nanohydroxyapatite solution and left at room temperature for ten minutes to react.

Then in polymer solution, solution of hydroxyapatite was added and the mixture put on the magnetic stirrer for 12 hours. Afterwards, 20 ml of the solution mixture was slowly poured on a polytetrafluoroethylene mold and left at room temperature for 12 hours to obtain the membranes. Next, the obtained membranes were immersed into the Sodium Hydroxide solution for ten minutes and washed repeatedly with distilled water until the membranes reached a neutral pH. Finally, the membranes were freezed and lyophilized below -50◦C for 24 hours at a pressure around 6mTorr using TFD5530 Bench top freeze dryer (IIShinBioBase, Republic of Korea) (Fig.2.1). The lyophilized membranes were stored at 4◦C in the refrigerator.

Characterization of The Membranes: SEM Analysis: To study the surface and cross section of the membranes scanning electron microscope (Mira 3 Tescan) was used. The specimens measuring approximately(100mm²) were cut from the fabricated membranes and were fixed on the stubs using double sided adhesive tape. Then the samples were coated with gold by using a gold sputter coating machine. SEM images were obtained at magnifications of 25kx and 50kx using secondary electrons at an accelerating voltage of 20kv and 10kv.

Bactericidal Activity of the Membranes: Invitro antimicrobial ability of the nanohydroxyapatite membranes was observed against S. Aureus (ATCC no.6538) and E. coli (ATCC no.14028) provided by the microbiology department of Riphah institute of pharmaceutical sciences Islamabad. The antimicrobial activity was performed by using a procedure called disc diffusion method[16]. For this Muller Hinton agar plates were used. The molten agar around 15 ml was poured into the sterile petri plates. After the solidification of

the agar, the cultured bacteria were spread with the help of sterile swab sticks onto the surface of the agar. The disc shaped membranes of 5mm diameter were then placed onto the media plates and kept in an incubator (Model B-53 IRmeco) at a temperature of 37◦C for a period of 24 hours. The inhibition zone was examined around the discs after the incubation, which was measured with a ruler in millimeter. The invitro antibacterial activity was performed in triplicate and results were presented as mean ± standard deviation.

RESULTS

The surface of the fabricated membranes observed through SEM at different magnification of 50kx (Fig.3.1a) exhibited a smooth and regular surface.
The cross section of the fabricated membranes observed at magnification of 25kx (Fig.3.1b) showed an uneven porous structure with some interconnectivity. The nHA and ACNs are well blended within the matrix of polymer.

Figure No.2.1: Showing the Fabricated Nanohydroxyapatite/Chitosan Membranes

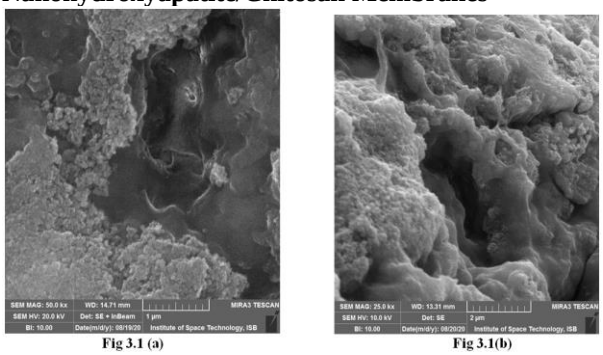

Figure 3.1(a) SEM images showing the surface of the membranes exhibiting a smooth and regular structure and Figure 3.1(b) showing cross section exhibiting porous structure.

Bactericidal Activity of the Membranes: The bactericidal ability of the membranes was assesssed for S.aureus(Plate A) and E.coli *(*Plat*e* B*)*.The area of inhibition formed againt S.Aureus after 24 hrs was 24.6± 0.4 and for E.coli it was 22.3±0.4 (table 3.1) indicating the antimicrobial action of the fabricated membranes as seen in (Fig.3.2) below. On the whole, the area of inhibition indicated satisfactory antibacterial activity of the fabricated membranes against both the strains.

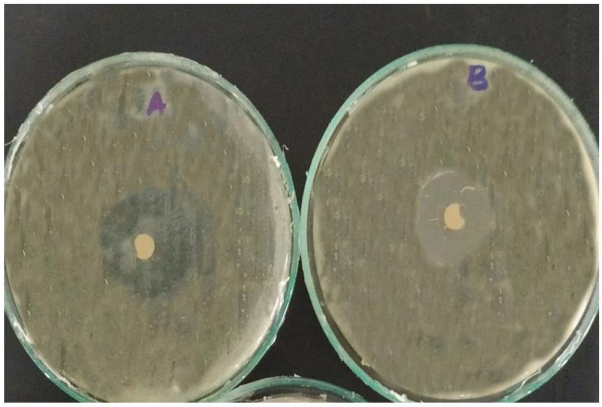

Figure No.3.2: Plate A and plate B showing antibacterial activity of the membranes against S. Aureus and E. coli

Table No.1: The zone of inhibition obtained are represented below

Organisms	Zone of inhibition (mean±s.d)
S.aureus	24.6±0.4 mm
E.coli	22.3±0.4 mm

DISCUSSION

The surface of the fabricated membranes observed through SEM displayed a smooth surface. The nHA and ACNs seem to be well blended within the matrix of polymer chitosan. The cross section of the fabricated membranes showed a porous structure with some possibility of interconnectivity throughout the structure. Although quantitative analysis of pore size could not be performed because of the unavailability of the software image J, however, a range of pore size was observed which could be advantageous as the studies have suggested that the small pores are beneficial for the mechanical integrity while the large pore size assists in migration of cells and in the carrying of the nutrients[17]. These pores are due to the effect of the lyophilization procedure performed in the fabrication of membranes in the current study where any solvent present in the matrix is removed resulting in a porous structure. This observation is in agreement with the previous studies[15].
The most commonly observed phenomenon in GBR/GTR application is the exposure of the membranes which leads to severe infections as it provides an environment for the adherence and

multiplication of the microorganisms [18].The results indicated satisfactory antibacterial activity of the membranes prepared in the study against both the strains. This target was achieved by the nanoparticles of Amoxicillin. The chief constituents of the gram positive bacterial cell wall are the linear chains of the peptidoglycan which is a polymer**.** Amoxicillin acts by inhibiting the cross linking of these chains. The gram negative microbes also have a cell wall made up of peptidoglycan which is surrounded by an additional external layer. This layer is thin in E. coli resulting in short cell membrane. The reason for the antimicrobial action against E.coli is this impaired semipermeable membrane which causes the death of the microbes [19].

Moreover, the antibacterial activity of the membranes can also be attributed to chitosan along with Amoxicillin, which is known for its broad-spectrum antimicrobial activity. This antibacterial effect is due to the reactions between the positively charged chitosan and the anionic membrane. As a result of these interactions there is release or interlocking of the cellular components. The positive charge of the chitosan molecules enables it to readily bind with the wall of the bacteria. This is because of the interaction among the cationic amino group at C-2 site and the glucosamine with the carboxylate of the polymer comprising the exterior of the bacterial cell. This interaction results in the formation of the polyelectrolyte complexes. These complexes form an impermeable membrane around the cell. Since this membrane is impermeable, it does not allow nutrients to reach the cell thereby inhibiting the metabolic activity of the bacteria. This membrane can also cause leakage of the cellular components by puncturing the cell wall[20].

The capability of chitosan to be effective against numerous microorganisms causing periodontal diseases has been documented previously [21]. It is also documented in literature that particularly the low molecular weight chitosan has an antibacterial activity against gram positive and gram negative bacteria [22]. Similarly, low molecular weight chitosan used in the current study for the fabrication of membranes is suggestive of the antibacterial activity. Therefore, it can be predicted from the results that the amoxicillin loaded membranes will provide antibacterial coverage in vivo as well.

CONCLUSION

The current study showed that the fabricated membranes displayed adequate morphology and antibacterial potential. Therefore, it is concluded that the fabricated membranes could be promising GTR/GBR membranes to be used for periodontal tissue regeneration applications.

Recommendations: In order to use these membranes for regenerative applications future research should be conducted to evaluate the mechanical properties, bioactive potential and cytotoxicity of the membranes.

Acknowledgement: We thank Dr. Shahid Amin and Dr. Mujahid of AMTECH Islamabad for providing nanohydroxyapatite to be used in the study.

Author's Contribution:

Concept & Design of Study:	Nausheen Ashraf
Drafting:	Maryam Saeedullah, Bakhtawar Yaqoob
Data Analysis:	Bakhtawar Yaqoob, Maryam Saeedullah
Revisiting Critically:	Nausheen Ashraf, Maryam Saeedullah
Final Approval of version:	Nausheen Ashraf

Conflict of Interest: The study has no conflict of interest to declare by any author.

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