

CHITOSAN - COPPER / STRONTIUM COMPOSITE MEMBRANE FOR BONE REGENERATION APPLICATION

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ABSTRACT:

The major source of chitosan (CS), a partly deacetylated version of chitin, is the exoskeleton of crustaceans. Due to its availability, adaptability, and distinctive qualities, such as biodegradability, biocompatibility, non-toxicity, hydrophilicity, anti-bacterial and anti-fungal characteristics, and wound-healing benefits, it stands out among other biomaterials. Since a few decades ago, chitosan-based bioactive polymers have been extensively employed in Bone regeneration. The well-known areas of tissue engineering utilising chitosan for the creation of biomaterials include cartilage, bone, intervertebral disc, blood vessel, corneal regeneration, skin, tissue fixation, and periodontal tissue engineering. To evaluate Chitosan - copper / strontium composite membrane for bone regeneration application. Chitosan (Cs) (from Shrimp Shells, degree of deacetylation $\geq 75\%$) and collagen (Col) Type I, Insoluble (from Bovine Achilles Tendon) were purchased from Sigma-Aldrich. Hydroxyapatite (HA) was synthesised in our laboratory following the reaction: $10\text{Ca}(\text{OH})_2 + 6(\text{NH}_4)_2\text{HPO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 12\text{NH}_4\text{OH} + 6\text{H}_2\text{O}$. Chitosan membranes (Cs) were prepared by solvent casting method. Chitosan solution was obtained by dispersing 1.5% (w/v) in an aqueous solution of acetic acid at 2% (v/v). The solution was mechanically stirred until solubilization. After that, 25 g of solution was poured into Petri dishes (diameter = 8.2 cm) and dried at room temperature. By using the solvent casting approach, composite membranes made of chitosan and collagen, chitosan and hydroxyapatite, and chitosan and collagen and hydroxyapatite were successfully created.

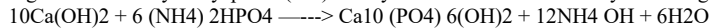
Keywords: Infectious disease, sexually transmitted disease, Communicable disease, medicine.

1. INTRODUCTION :

The major source of chitosan (CS), a partly deacetylated version of chitin, is the exoskeleton of crustaceans. Due to its availability, adaptability, and distinctive qualities, such as biodegradability, biocompatibility, non-toxicity, hydrophilicity, (1) anti-bacterial and anti-fungal characteristics, and wound-healing benefits, (2) it stands out among other biomaterials. Since a few decades ago, chitosan-based bioactive polymers have been extensively employed in tissue engineering. (3) The well-known areas of tissue engineering utilising chitosan for the creation of biomaterials include cartilage, bone, intervertebral disc, blood vessel, corneal regeneration, skin, tissue fixation, and periodontal tissue engineering. (4) The biomedical sector is very interested in tissue engineering since it can repair many tissues, including bone, epithelial tissue, and cartilage. Tissue engineering attempts to eliminate the need to replace and repair damaged tissue in this setting. In order to stimulate, direct, and enhance the regeneration of various connective tissues, including skin, (5) cartilage, and bone, many efforts have been done (3,6). Researchers have come up with a number of techniques throughout the years for extracting chitosan from the shells of various crustaceans, insects, and fungus. Additionally, Labeo Rohita's leftover fish scales may be utilised to extract chitosan. With variable degrees of acetylation, chitosan has been widely extracted from chitin using two distinct techniques. (7) One of them is the homogeneous deacetylation of pre-swollen chitin under vacuum in an aqueous media, and the other is the heterogeneous deacetylation of solid chitin. Both times, the deacetylation reaction requires strong alkali solutions and extended processing durations. Due to the rise in bone problems caused by the ageing population, increasing obesity, and insufficient physical activity, bone repair medicine research has received a lot of interest. The bone tissue cannot repair on its own when the bone problem is larger than the critical size defect (>2 cm), necessitating therapeutic therapy (7). With more than two million bone graft surgeries performed each year globally, bone grafting is one of the most popular techniques for bone regeneration. In the last few decades, a variety of bone graft types have been employed in bone tissue engineering; however, more focus is now being placed on the biomimetic approach to scaffold design, which achieves molecular, structural, and biological compatibility with complex natural bone tissue. The properties of these materials open a wide range of possibilities in composite biomaterials considering tissue engineering and biomedical applications. Although strontium oxide works better, calcium and magnesium oxides are more commonly used. These drawbacks are brought on by the difficult preparation process and size control issues, and further study is needed to make SrO-based catalysts economically viable. To get around this obstacle, many scientists are currently working to create strontium oxide (SrO) nanoparticles using straightforward techniques like wet and bio-reduction methods, as well as examining their characteristics and potential uses in a range of products like gas sensor electrodes, lithium-ion batteries, semiconductors, and super capacitors.

2. MATERIALS AND METHOD :

Chitosan (Cs) (from Shrimp Shells, degree of deacetylation $\geq 75\%$) and collagen (Col) Type I, Insoluble (from Bovine Achilles Tendon) were purchased from Sigma-Aldrich. Hydroxyapatite (HA) was synthesised in our laboratory following the reaction:



Briefly, ammonium phosphate solution was applied dropwise to a $\text{Ca}(\text{OH})_2$ suspension in deionized water. The mixture was thoroughly stirred while being kept at a constant temperature of 25 °C. This solution's pH was raised to 10, and it was then let to stand at room temperature for 16 hours. The product was removed from the mother solution and rinsed repeatedly until the pH was neutral. The powder was produced and dried for 20 hours at 60 °C. By using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy, the final material was evaluated (FTIR).

3. RESULTS AND DISCUSSION :

Chitosan composite membranes formation

3.1 Chitosan membranes: Chitosan membranes (Cs) were prepared by solvent casting method. Chitosan solution was obtained by dispersing 1.5% (w/v) in an aqueous solution of acetic acid at 2% (v/v). The solution was mechanically stirred until solubilization. After that, 25 g of solution was poured into Petri dishes (diameter = 8.2 cm) and dried at room temperature (~a week).

3.2 Chitosan/collagen composite membranes: Chitosan/Collagen composite membranes (Cs/Col) were obtained varying collagen content into the chitosan solution. Briefly, collagen (0.15 and 0.75% w/v) was dispersed into a chitosan solution prepared as described above by mechanical stirring until a good collagen dispersion was obtained. Then, 25 g of solution was poured into Petri dishes and dried at room temperature.

3.3 Chitosan/hydroxyapatite composite membranes: Chitosan/hydroxyapatite composite membranes (Cs/HA) were obtained varying HA content into the chitosan solution. Briefly, HA (0.15 and 0.75% w/v) was dispersed into a chitosan solution prepared as described above by mechanical stirring until a good dispersion of HA was obtained. Then, 25 g of solution was poured into Petri dishes (diameter = 8.2 cm) and dried at room temperature (~a week).

3.4 Chitosan/collagen/hydroxyapatite membranes: Chitosan/Collagen/Hydroxyapatite membranes (Cs/Col/HA) were obtained, varying HA and collagen content into the solution. Briefly, HA (0.15 and 0.75% w/v) and collagen (0.15 and 0.75% w/v) were dispersed into a chitosan solution prepared as described above by mechanical stirring until a good dispersion of HA and collagen was obtained. Then, 25 g of solution was poured into Petri dishes (diameter = 8.2 cm) and dried at room temperature (~a week).

Figure 1: SEM Micrograph and Elemental Mapping of the Chitosan-Composite Membrane. Scanning Electron Microscopy (SEM) image showing the surface morphology and particle distribution within the membrane matrix. The white rectangular box indicates the specific area ("Spectrum 1") selected for Energy-Dispersive X-ray (EDX) analysis.

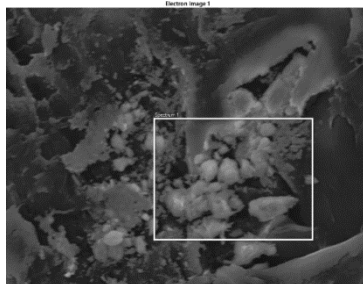


Figure 2: EDX Spectrum of the Chitosan-Copper/Strontium Composite. Energy-Dispersive X-ray (EDX) spectrum confirming the elemental composition of the membrane. The peaks and the data table verify the presence of Carbon (C) and Oxygen (O) from the organic chitosan/collagen matrix, along with Strontium (Sr) and trace Copper (Cu) dopants used for enhanced bone regeneration.

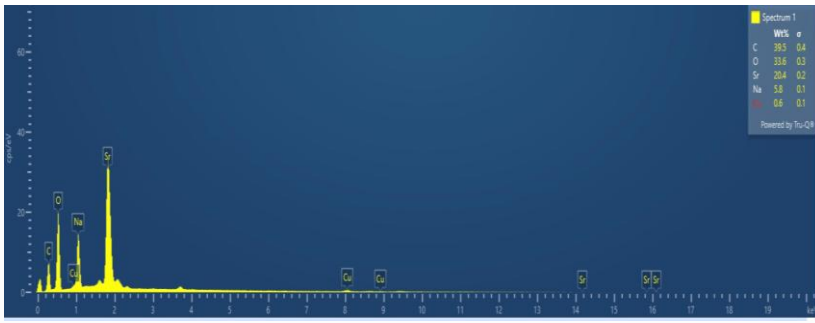


Figure 3: Low-Magnification SEM Surface Analysis. SEM micrograph at 140x magnification showing the general topography and uniformity of the solvent-casted chitosan membrane. The scale bar represents 50micrometre.



Figure 4: High-Magnification Morphological Characterization. High-resolution SEM image (1.90kx magnification) highlighting the micro-porous structure and surface roughness of the composite, which are critical factors for mesenchymal stem cell (MSC) attachment.

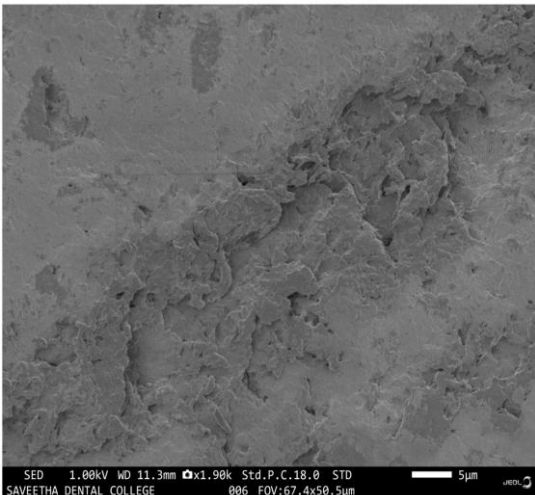


Figure 5: Cross-Sectional Fibrillar Structure. SEM micrograph at 800x magnification showing the internal fibrillar network of the chitosan/collagen/hydroxyapatite blend. The interconnected pathways are designed to facilitate nutrient transport and cell infiltration.

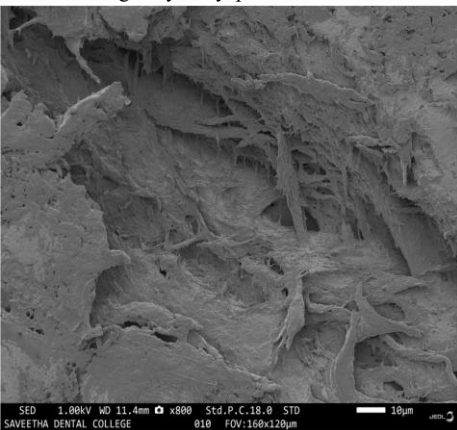


Figure 6: Surface Porosity and Nanopore Distribution. SEM analysis at 1.60kx magnification reveals the distribution of micro and nanopores produced by the hydroxyapatite dispersion within the organic matrix.

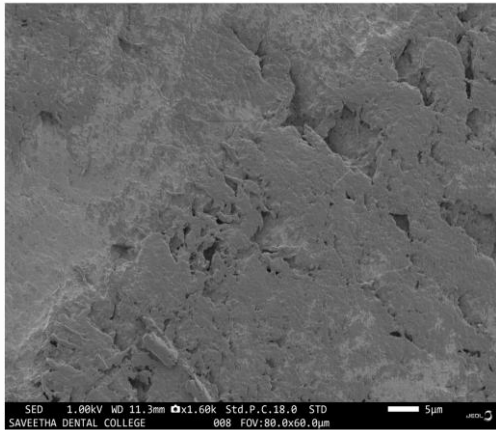
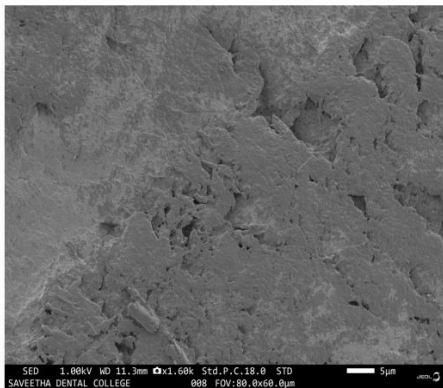


Figure 7: Microstructural Topography for Cell Adhesion. Detailed SEM view 1.60kx of the membrane surface. The observed topography supports the study's conclusion regarding high cell adhesion and the absence of cytotoxicity in the prepared materials.



FTIR analysis

To evaluate the possible interactions between the chitosan, hydroxyapatite, and collagen molecules, FTIR analysis was performed with a Perkin Elmer Spectrum100 equipment, using the Horizontal Attenuated Total Reflectance mode in the wavenumber range from 650 to 4000 cm^{-1} .

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was carried out by using a METTLER TOLEDO, TGA/DSC 1 STARe SYSTEM with a scan range from 25 to 600 $^{\circ}\text{C}$, a heating rate of 10 $^{\circ}\text{C}$ per min^{-1} , using air as reactive gas and nitrogen as protective gas, with a flux of 60 mL/min .

Indirect cytotoxicity test

We approached a hemolysis test as a first step to determining these membranes' biocompatibility because it is an easy and quick test to assess if these materials can be cytotoxic for other cell types [69].

Cytotoxicity of the different membranes was determined by the assay employing hemolysis of erythrocytes isolated from human blood, carried out with a PBS solution pre-incubated with the membranes for 48 h. We followed a modified protocol of Evans et al. [70]. Briefly, human blood was collected from three healthy volunteers, 6 mL of each patient's blood was placed in a heparinized tube and centrifuged for 5 min, and then the plasma was removed. A NaCl solution of 150 mM was used to fill the tube and further centrifuged for another 5 min. The supernatant was removed and discarded. A second wash step with PBS was followed and then a solution of 1:50 erythrocytes: PBS was prepared.

To obtain a "PBS conditioned solution", membranes were placed in a PBS solution and incubated for 48 h at 37 $^{\circ}\text{C}$.

For hemolysis assay, 10 μL of "conditioned PBS solution" was mixed with 190 μL of erythrocytes solution and incubated for 1 h at 37 $^{\circ}\text{C}$. The hemolysis was detected through spectrophotometric measurement of the supernatants of red blood cells treated with experimental agents at 492 nm. Additionally, as a positive control, we use 10 μL of Triton X-100 20% and a solution of 10 μL of non-conditioned PBS in 190 μL of erythrocytes solution as the negative control.

Cell viability assay

Cell viability was quantified using the commercial kit CellTiter 96 Aqueous One Solution Cell Proliferation Assay® (Promega). Following the trader's instructions. For this assay a tetrazolium salt, named MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium] is used. The MTS is reduced by cells to formazan, a colored product soluble in an aqueous solution, in a culture medium. This conversion is carried out by mitochondrial dehydrogenase enzymes present in viable cells in the culture. Here, the amount of product formed (formazan), determined from the absorbance at 490 nm is directly proportional to the number of viable cells in the culture.

To determine the cell viability of MSC attached on the membranes, we perform the assays using hBFP-MSCs. 4000 cells were seeded in each of the 96 well-plate, were previously placed the different composite membranes and cultured with α -MEM + 10% SFB for 44 h before. After that, 20 μL of culture medium was replaced with 20 μL of CellTiter 96 Aqueous One Solution Cell Proliferation Assay® reagent in each well and left 4 h under incubation. The medium was removed, and the absorbance in each well was measured in a microplate reader set to 490 nm. The amount of product formed is directly proportional to the number of viable cells in the culture. The reduction of MTS achieved by control (cells grown directly on the plate) was set at 100%, and cultivate cells over the membranes were expressed as a percentage.

SEM analyses

The morphological characterization of the different synthesized samples and the seeded membranes was carried out in a Scanning Electron Microscopy (SEM) FEI Inspect F50 System attached with an Energy-Dispersive Spectrometer (EDX) EDAX Apollo.

The preparation of the seeded membranes for SEM analyses was performed modified method. Briefly, samples were fixed using 2.5% v/v glutaraldehyde in PBS for 1 h at 4 $^{\circ}\text{C}$, then postfixed with 1% v/v OsO_4 in PBS for 1 h at 4 $^{\circ}\text{C}$ and rinsed three times with distilled water. After, seeded membranes were dehydrated with a graded series of ethanol (50, 70, 80, 90, and 100% v/v), followed by a 5 min incubation with hexamethyldisilazane (HMDS, Sigma-Aldrich®) and left at room temperature for 2 min to dry. The samples were carbon/Pt coated in Balzers BA 510 evaporator.

CONCLUSION :

By using the solvent casting approach, composite membranes made of chitosan and collagen, chitosan and hydroxyapatite, and chitosan and collagen and hydroxyapatite were successfully created. Containing good hydroxyapatite dispersion in the organic matrix, membranes with micro and nanopores were produced. The thermal stability and thermal breakdown of the composites are improved by the addition of collagen and hydroxyapatite to chitosan. The highest cell adhesion

was demonstrated by the membranes with the highest hydroxyapatite and collagen contents, and none of the produced membranes displayed any cytotoxicity, indicating that these materials have a significant potential for use in bone regeneration.

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