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Original Article

Development and characterization of Alginate-nanochitin composites for wound healing application

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ABSTRACT

Lyophilized sodium alginate dressings were fabricated with and without the incorporation of nano chitin as filler. Nano chitin prepared using a combination of acid hydrolysis and probe sonication had average particle size of 13.5 ± 11.8nm with a polydispersibility index of 0.005. Lyophilization resulted in dressings with soft and spongy texture with excellent folding endurance that could be conveniently applied over wound surface. The thickness of the dressings ranged from 1.22 to 2.05mm.with pH between 6.9 and 7.3. Tensile strength of the dressings ranged from 0.029 to 0.039 N/mm^2 and was found to increase with increasing polymer concentration. The incorporation of nanochitin into the alginate matrix resulted in greater tensile properties, which is a desirable parameter in wound dressing materials. The WVTR was found to be from 722.9 to 1007.5 g/m^2.24h. The *in-vitro* cytocompatibility study conducted on L929 mouse fibroblast cell lines proved that the dressings were non-toxic and suitable for wound healing application.

Keywords: Wound dressing, chitin, alginate, lyophilization

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INTRODUCTION

Chronic cutaneous wounds and ulcers represent a significant portion of hospital admissions, and the healing of these wounds is typically a costly and time-consuming endeavor. An ideal dressing should maintain a moist and protective healing environment and yet be highly absorbent. The dressing should be soft, flexible, and should not reinjure the wound upon removal.[1] Lyophilized dressings are an established drug delivery system for application to suppurating wounds. They can absorb wound exudates and get converted into a gel, offering a moist environment that is vital for wound healing.[2] Chitin, the second most abundant polymer in nature, is a renewable, nontoxic, biodegradable and antibacterial polysaccharide.[3] Nano-sized chitin whiskers can be incorporated into polymer films for obtaining higher crystallinity and rigidity. It can also elevate the composite film's mechanical property when used as a filler material.[4] Moreover, chitin has been reported to have antibacterial, wound healing and tissue regeneration properties.[5] The present work reports the fabrication and evaluation of chitin incorporated alginate dressings for potential wound healing application.

MATERIALS AND METHODS

Chitin was obtained from Marine Chemicals, Cochin; Sodium alginate was procured from Yarrow Chem Products, Mumbai. All other reagents used were of analytical grade.

Preparation of nanochitin: Nano chitin was prepared using the method reported by N. Murthy *et. al.*[6]with some modification. 10grams of commercial chitin flakes was added to 75ml of 12M conc. HCl. The mixture was stirred at 200 rpm for 1hour at room temperature using a mechanical stirrer. The solution was filtered using glass wool to remove any undissolved residue. The filtrate was added to one litre ice cold water in order to obtain colloidal precipitate of chitin. The product was left at 4^0C for 8 hours to facilitate the precipitation process. The precipitate was collected by vacuum filtration and washed with deionized water until the filtrate was neutral (pH 7.0). The moist colloidal chitin was dried at 50^0C for 12h.

Particle size analysis of nanochitin by Dynamic Light Scattering: The size of the chitin nanofiber hydrocolloid was studied by Dynamic Light Scattering using NanoPlus, Particle Size & Zeta Potential Analyzer. The light source was operated at a wavelength of 633nm.[7]The chitin nanofiber aqueous suspension was diluted to a concentration of 0.1% w/v and filled in a PMMA (Poly Methyl Methacrylate) cuvette and scanned three times at ambient conditions (i.e., 25°C).

Preparation of nanochitin alginate composites
The alginate and alginate-nanochitin composite films were prepared according to formulations showed in Table 1.

Table 1: Formulation of sodium alginate films

Ingredients	SA-1	SA-2	SA-3	SAC-1	SAC-2	SAC-3
Sodium alginate (% w/w)	2.0	2.5	3.0	2.0	2.5	3.0
Nano chitin (% w/w)	-	-	-	1.0	1.25	1.5
Glycerol	40% w/w of polymer					

SA – Sodium alginate dressing
SAC- Nano chitin incorporated Sodium alginate dressing

Nano chitin was dispersed in deionized water and subjected to probe sonication at the following conditions: Amplitude: 40%; Pulse: 5 sec on, 5 sec off and duration 15 min. The weighed amount of sodium alginate and glycerol was then added to the colloidal chitin using a mechanical stirrer operated at 100 rpm for 1h. The formulation mixture (20g) was casted on petri plates (9cm diameter).

The casted mixture were frozen overnight at -40^0C and subjected to freeze drying (Martin Christ, ALPHA 1-2 LD plus) for 8 hours.

Evaluation Studies
Thickness: The batch film thickness was determined by a screw gauge and recorded as the mean of five measurements representing four corners and the center of each batch film.[8]

Folding endurance: Folding endurance was determined to find the flexibility of film which is needed to handle the film easily and for comfortable, secured application of film on the wound. It was determined by repeatedly folding the sample film at same place till it breaks or folded

upto 300 times manually. Number of times the film could be folded at the same place without breaking gives the value of folding endurance.[9]

Tensile strength and elongation at break: Tensile strength measures the ability of film to withstand rupture, mechanical pressures or the force required to break the film.[10]Tensile strength of the film was determined by using a fabricated tensile testing instrument. It consists of two grips. The lower one is fixed and upper one is movable. The test film of specific size was fixed between these cell grips and the weight was incremently applied on the movable grip. The weight required to break the film was recorded and converted into equivalent force in Newtons. Tensile strength (N/mm^2) was then measured using the length and width of the film (mm).

$$Tensile\ strength = \frac{Weight\ at\ break}{Surface\ area\ of\ the\ film}\ X\ 0.0098$$

Elongation at break was calculated using the following formula

$$Percentage\ \ Elongation\ at\ break = \frac{Length\ at\ break - Initial\ Length}{Initial\ length} \times 100$$

Water vapour transmission rate: The dressing ability to control water loss from the surface of the wound can be determined by the WVTR. The wound surface moisture can be regulated by the use of various wound dressings with different WVTRs. An extremely high WVTR may lead to dehydration of a wound, whereas an unacceptably low WVTR may cause the accumulation of wound exudates. Hence, a dressing with a suitable WVTR is required to provide a moist environment for natural healing.[11]

To determine moisture permeability of the membranes, the WVTR was measured according to the American Society for Testing and Materials (ASTM) standard.[12] Briefly, a sample was cut into a disc and mounted on the mouth of a cylindrical cup containing distilled water (donor compartment). The sample and cup were sealed with Teflon tape across the edge and then placed into a 37°C environmental chamber at 50% RH. The samples were weighed after 24h. All measurements were repeated three times (n = 3). The water vapour transmission rate (g/m^2.24h.) was determined using the formula:

$$WVTR = \frac{\Delta W}{\Delta T.A}$$

ΔW is the change in weight of the donor compartment
ΔT is the time = 24 hours
A is the surface area of the film available for permeation

Percentage porosity: The sample was cut into square shape and the length, width and thickness of the sample was measured using Vernier calipers to calculate the volume (V). The sample was then weighed (W1) and subsequently immersed in absolute ethanol. The sample was weighed again (W2) after it was saturated.[11] The porosity was calculated as:

$$Porosity = \frac{W2 - W1}{\rho V}\ X\ 100$$

Surface morphology of nano chitin and composite films by Scanning Electron Microscopy: The output of electron microscopy is a result of the interaction of the sample with electron beam. Many factors such as electron energy, sample density, atomic number of elements and topography of the sample surface, have an effect on this interaction.

The Scanning Electron Microscopy was carried out at IISc., Bangalore using Scanning Electron Microscope with EDAX attachment (Cambridge CF). The sample surface was covered with a thin layer of gold to avoid a repulsive reaction of electron beam. The nano chitin and lyophilized composite films were scanned at a magnification of 500X and 1000X. The surface morphology of the lyophilized films with and without incorporation of nano chitin was obtained. The effect of nano chitin incorporation on the surface morphology of the films was determined.

***In vitro* cytocompatibility:** *In vitro* cytotoxicity test of sodium alginate composite films were performed using mammalian mouse fibroblast cell line L929 by direct contact method at Genelon Institute of Life Sciences, Bangalore as per ISO-10993-5 guideline. L929cells were used in the present study, because it can be easily cultured in a reproducible manner, and also this cell line has been widely used for preliminary cytotoxicity evaluation for a wide range of biomaterials because of easy proliferation and adherence on most of the biomaterial surface.

The L929 cells were subcultured, trypsinized and seeded on to multiwall tissue culture plates. The L929 fibroblast cells were cultured with Dulbecco's Modified Eagle Medium, (DMEM-High Glucose #AL111, Himedia) 10% Fetal Bovine Serum (#RM10432, Himedia) and incubated at 37^0C in 5% CO_2 atmosphere for 12h. untill formation of the cell monolayer. The test specimen was incubated (in concentrations of 25%, 50%, 75% and 100%) with mono-layer cells at 37^0C for 24 hours in a 5% CO_2 atmosphere. The cell monolayer was examined using inverted microscope (Biolink) for cellular response. *In vitro* cytotoxicity of the test specimen was compared with the negative control (medium with cells but without the experimental drug/compound), and positive control (medium with cells and with 25uM of Camptothecin), a toxic material.

***In vitro* Cell viability study:** The MTT assay was performed to measure the metabolic activity of cells and estimated through 'color-change' phenomenon from yellow colored tetrazolium salt, MTT {3-(4, 5-diamethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide} to purple colored formazan[13].

The cultured cells from the cytocompatibility study were treated with MTT reagent to a final concentration of 0.5mg/mL of total volume and further incubated at 37 ± 1^0C for 3 hours in humidified and 5% CO_2 atmosphere. The excess amount of MTT was removed by aspiration and 100µl of DMSO added in order to dissolve the formazan crystals. Gentle stirring in a gyratory shaker was carried out to enhance dissolution. The color exchange was quantified by measuring absorbance at 570 nm using a ELISA plate reader (ELX-800 Biotek).

To calculate the reduction of viability compared to the blank, the following equation was used

$$Percentage\ Viability = \frac{Optical\ density\ of\ the\ test}{Optical\ density\ of\ the\ blank} \times 100$$

RESULTS AND DISCUSSION

Preparation of nanochitin: Nano chitin was prepared using combination method of acid hydrolysis[14] and ultrasonication[15] techniques. Acid hydrolysis dissolves water insoluble, crystalline residues in chitin and converts it into a colloidal suspension. It also causes protonation of the amino groups and stabilizes the colloidal dispersion. Probe sonication further helps in achieving reduced particle size and uniformly dispersed particles.

Particle size analysis of nanochitin by dynamic light scattering: The polydispersity index is a measure of the width of molecular weight distributions. Poly dispersibility values greater than 0.7 indicate that the sample has a very broad size distribution. The size of the colloidal chitin was estimated based on DLS data. The average particle size of the nano chitin was found to be 13.5 ± 11.8 nm with a poly dispersibility index (P.I) of 0.005 (Fig. 1) indicating a narrow size distribution. The intensity distribution table indicates that 90% of the particles fall within 29.80 nm.

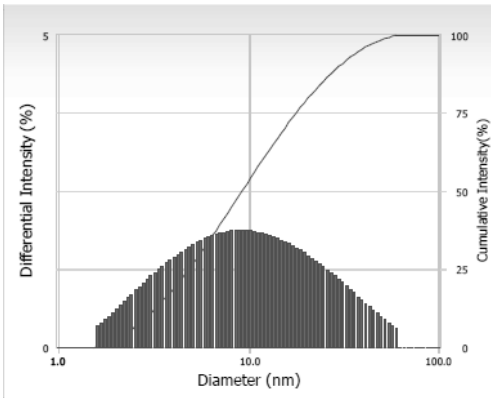


Fig.1: DLS of Nano chitin showing intensity distribution

Evaluation of Nano Chitin-Alginate Composites

Surface pH: The surface pH of all the films ranged from 7.1 to 7.4. Since the pH of the films were near neutral, the films could be considered suitable for application onto the wounds.

Thickness: The thickness of the dressings ranged from 1.22 - 2.05mm. Lyophilization resulted in spongy films with air entrapment, thus resulting in increased thickness. Also small increase in thickness of the film was observed as the concentration of the polymer was increased in the formulation.

Folding endurance: The values of more than 300 indicated excellent folding endurance property of the films for wound application.

Tensile strength: The tensile strength of the composite films with nano chitin was found to be higher compared to films without chitin indicating improvement of tensile properties of the films with

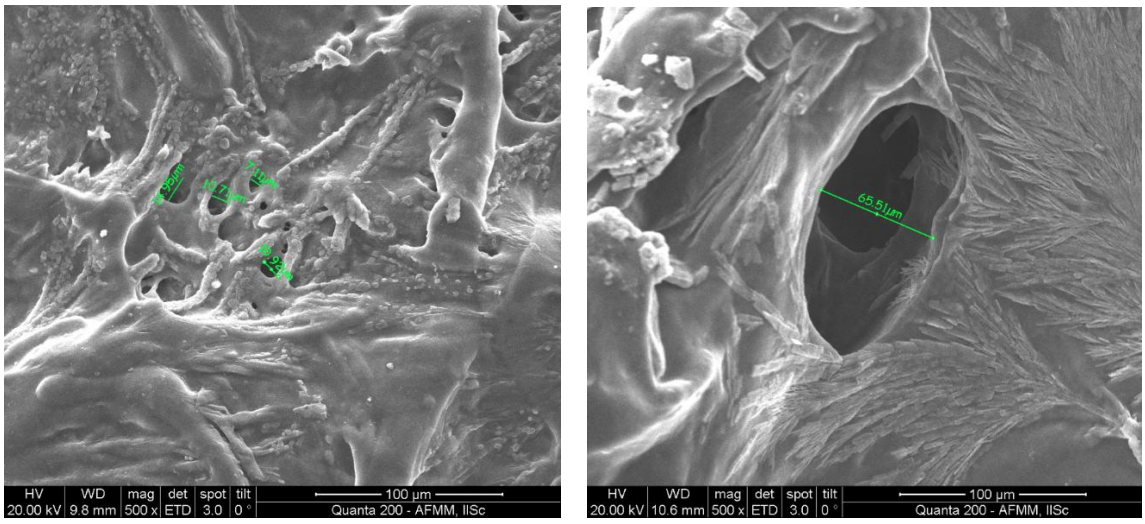
incorporation of nano chitin in the polymer matrix. Due to their size, mechanical strength and relevant biological properties, nano chitin has been majorly applied as nanofillers in the reinforcement of both natural and synthetic polymer composites. The studies on tensile strength supported this finding.

Water vapour transmission: The WVTR of the dressings was found to be in the range of 722.9 to 1007.5 g/m^2.24h. The values suggest that dressings will be able to transmit water vapour in order to maintain an optimum moist environment on the surface of the wound. This property prevents excessive drying out of the wound surface and also prevents wound maceration due to accumulation of wound fluids.

Table 2: Evaluation parameters for the dressing

Evaluation Parameters	SA-1	SA-2	SA-3	SAC-1	SAC-2	SAC-3
Thickness (mm)	1.22±0.015	1.75±0.032	1.97±0.07	1.31±0.025	1.83±0.0152	2.05±0.037
Folding endurance	>300	>300	>300	>300	>300	>300
Tensile strength (N/mm^2)	0.029±0.003	0.030±0.001	0.035±0.002	0.031±0.002	0.034±0.001	0.039±0.001
% Elongation at break	24±2.56	30±2.98	35±3.22	21±1.52	24±1.98	29±2.08
% Porosity	189.67±0.32	76.43±0.47	69..01±0.78	192.89±0.39	83.53±0.93	74.79±0.56
WVTR g/m^2.24h	722.9±0.032	889.8±0.048	988.7±0.049	820.9±0.029	1007.5±0.076	970.1±0.066
Surface pH	7.3±0.001	7.1±0.002	6.9±0.00	7.0±0.002	7.3±0.001	7.3±0.002

Surface morphology of nano chitin and composite films by Scanning Electron Microscopy



A **B**

Fig. 2: SEM photomicrographs of sodium alginate films without (A) and with (B) nano chitin at 500X

The SEM images of dressings without and with nano chitin is shown in Fig.2. Lyophillization method of drying the films resulted in softer, thicker and porous films in contrast to films dried using tray drier. The porous morphology was clearly indicated in the SEM photomicrographs. The alginate films without chitin are seen to be more uniform and having a smoother morphology. The composite alginate films with chitin showed the fibrous dispersion of chitin in their matrix indicating a uniform dispersion of chitin. The pore size in the alginate films without chitin is smaller compared to the pore size in films with chitin. This may be due to the oppositely charged nature of alginate and chitin.

***In vitro* cytocompatibility:** *In vitro* cytocompatibility assessment was carried out on

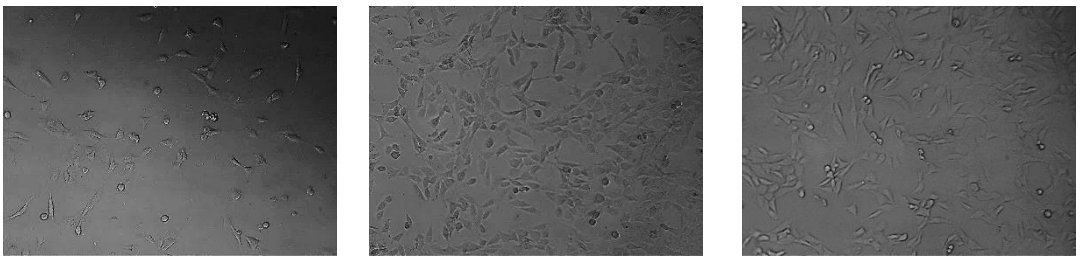
L929 mouse fibroblast cells. The cell morphology was visualized after 24hours incubation using a phase contrast microscope. Phase contrast microscopic images revealing the adhesion of cultured L929 cells after one day of incubation are shown in Fig.3.

The cells on negative control have typical fibroblast-like morphology with cell-to-cell contacts and filopodia extension. In contrast, cell density on positive control is decreased with a globular morphology indicating cell death. The cells exposed to the composite dressings exhibit similar morphology as the negative control with the filopodia extension.

In the MTT assay, the developed formazan is directly proportional to the number of mitochondrially active cells. The optical density of the purple coloured formazan is measured at 570 nm using ELISA plate reader. The optical density readings are reported in Table 3. A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed, as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank, the following equation was used

$$\%\ Viability = \frac{Optical\ density\ of\ the\ test}{Optical\ density\ of\ the\ blank} \times 100$$

The lower the % viability, higher the cytotoxic potential of the test. If viability is reduced to less than 70 % of the blank, it has cytotoxic potential.



Positive control **Negative control** **Sodium alginate chitin composite**

Fig. 3: Phase contrast microscopic images of L929 mouse fibroblast cells subjected to cytocompatibility studies.

Table 3: Cell viability studies – Formazan Absorbance at 570 nm

Sl. No.	Positive control	Negative control	Chitin-alginate composite
1	0.4705 ± 0.0106	0.9095± 0.0035	0.883 ± 0.0084

n = average of three readings ± S.D

The formazan absorbance of the composites in comparison to positive and negative controls is given in table 3. The viability of cells exposed to the composites was found to be comparable to negative control. The study showed the suitability of the composites for application onto wounds and indicated that the dressings did not possess any cytotoxic potential.

CONCLUSION

Nano chitin was prepared using modified method by combining acid hydrolysis with probe sonication technique and incorporated as filler in the development of nanochitin-alginate composite films.The lyophilised dressings exhibited porous morphology as observed in the SEM images. This also facilitates the rapid absorption of the wound fluids and maintaining the necessary moisture required for optimum wound healing. The lyophilized chitin incorporated alginate dressings met the technical specifications of tensile strength, water vapour transmission and pH needed for wound care fabric. The *in-vitro* cytocompatibility studies indicated the suitability of the dressing for wound healing application without cytotoxic potential.

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