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Fabrication of Layer by Layer Sandwich Composite using Biopolymer and Antibacterial Agent

Christophe Bizimana, Maleeka Begum, Rajesh and Srinivasan

Research Student, Department of Biotechnology, Sri Krishna Arts and Science College, Tamil Nadu, India.

Head and Professor, Department of Biotechnology, Sri Krishna Arts and Science College, Tamil Nadu, India.

Research Scholar, Department of Biotechnology, Sri Krishna Arts and Science College, Tamil Nadu, India.

Assistant Professor, Department of Biotechnology, Sri Krishna Arts and Science College, Tamil Nadu, India.

ABSTRACT: Natural polymer, are polymers derived from living creatures. Collagen, Chitosan, Oligochitosan, with silver nanoparticle ($AgNO_3$) and plant medicine (herbal extraction), were used in the study for the formation of biofilm layer which acts as the base for development of fibroblast cells which can be an replacement of artificial cell line in case of wound healing. The biopolymer films were prepared with blend of poly vinyl alcohol in which active ingredient such as metal nanoparticles or herbal drug has been loaded. Layers of films were prepared with each polymer blended with poly vinyl alcohol. Each of the single film was multilayered with gelatine as adhesive. Antibacterial activity of the prepared film was tested against nosocomial pathogen by disc.

KEYWORDS: Collagen, Chitosan, Oligochitosan, Silver nitrate, *Herbal nanoparticles*

I. INTRODUCTION

Since 1980s, some scientists have been using collagen as a matrix to regenerate tissues for repairing skin, bone, joint cartilage, oesophagus, muscle and nervous system. The use of collagen combined with glycosaminoglycans as a skin implant has been already tested. The ability of collagen gel to regenerate cornea and nerves has been also demonstrated by recent animal studies and clinical trials [14]. Furthermore, it has been shown that the combined collagen and hyaluronic acid can promote the revascularization of tissues in animal models. In the beginning of 1970s and 1980s the research on collagen was initiated by interested scientists and commercial research laboratories expanding medical applications of biomaterials and connective tissue research. Collagen plays a significant role in the formation of organs and tissues, and is involved in different functional expressions of cells. Many natural polymers and their synthetic analogues are used as a biomaterial, but the characteristics of collagen as a biomaterial are different from those of synthetic polymers mainly in its mode of interaction inside the body. The important features of collagen are its biocompatibility, biodegradability and weak antigenicity [15]. In the body as compared with other natural polymers like gelatine and albumin, Collagen possesses good ability to penetrate a lipid-free interface. The primary reason for the usefulness of collagen in biomedical application is that collagen can form fibres with extra strength and stability through its self-aggregation and cross linking. Collagen is the most abundant protein constituting to the 30% of total protein and 6% of animal body weight. Type I collagen, a natural polymer, is a major extracellular matrix protein in mammals and exhibits favourable characteristics for promoting cell proliferation. It can influence the cell physiology and morphology, create a good matrix for endothelial cells *in invitro conditions*, induce platelet aggregation, promote blood clotting, and consequently accelerate the healing of skin wounds [11]**.** Basically collagen is a naturally existing protein present in the animal body, fibrous in nature, and especially found in the connective tissue and flesh of mammals. Approximately 25% - 35% of total body protein is comprised of collagen, in the form of elongated fibrils; collagen is abundantly present in fibrous tissue like bone, cartilage, tendons, blood vessels, ligament, skin, cornea, inter-vertebral disc and the gut. The synthesis of collagen in the body is made by fibroblast cells. Collagens possess good tensile strength, and found both. outside and inside the body cells. In combination with elastic, collagen provides support to body tissues and organs, basically collagen offers firmness and strength and elastic provides flexibility to

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body tissues. In fact gelatine which is used in food and pharmaceutical industries is collagen that has been hydrolysed irreversibly [16].

Structure of collagen: Basically collagen possesses a triple helix structure, which generally made up of two homologous chains (α-1) and one supplementary chain that varies slightly in its chemical composition (α-2). These chains are polypeptide in nature and coiled around one another in a cable form. Each has a distinct turn in the reverse direction, these chains are connected together chiefly by hydrogen bonds between nearby CO and NH groups. The weight of collagen molecule is 300 kda and its structure is rope shaped and having a length of 300 NM and a width of 1.5 NM. The major content of glycine and amino acid residue is affecting the helix formation; in each of three chains of collagen molecule the amino acids are regularly arranged. The sequence of amino acids follows the pattern glycine-proline-X or glycine-X-hydroxyproline where X is the amino acid other than glycine, proline or hydroxyproline; glycines constitute about 1/3 of total sequence and proline or hydroxyproline accounting for the 1/6 of the sequence. This whole structure is joined with the help of hydrogen bonds and linking peptide bonds [6].

Collagen can be reabsorbed into the body, is non-toxic, produces only a minimal immune response (even between different species), and is excellent for attachment and biological interaction with cells [2]. Collagen may also be processed into a variety of formats, including porous sponges, gels, and sheets, and can be cross linked with chemicals to make it stronger or to alter its degradation rate. The number of biomedical applications in which collagen have been utilized is too high to count here it not only has been explored for use in various types of surgery, cosmetics, and drug delivery, but in bio prosthetic implants and tissue-engineering of multiple organs as well. Cells grown on collagen often come close to behaving as they do within the body, which is why collagen is so promising when one is trying to duplicate natural tissue function and healing [2]. A method for constructing a film by using poly (hydroxybutyratevalerate) (PHBV) grafted with scaffold type I collagen to support silver nanoparticles (AgNPs) [17]. A blend of *Aloe Vera* with collagen and chitosan scaffold for tissue engineering applications. To fabricate a dermal equivalent tri-layer composite to treat wound infections using a natural polymer and antibacterial [18].

Even though the mammalian body retains a plenty amount of collagen, those tissues rich in fibrous possess collagen such as skin and tendons, are commonly used as preliminary materials to produce collagen for use in transplants, wound dressings, or drug delivery systems. In addition procaine, bovine and sheep collagen varieties derived from many different sources including marine sources, human placenta, and recombinant human collagen from transgenic animals must be labelled. Autologous collagen material deals additional gut alternative mucosa which is consumed in the building of surgical sutures. Collagen is insoluble in organic solvents. Water-soluble collagen denotes only a minor fraction of total collagen and the quantity depends on the age of the animal and kind of tissue extracted. In certain tissues, especially the skin of young animals, cross linking is sufficiently little to extract a few percent in suitable conditions. Still, collagen molecules present inside fibril masses can be separated and brought into aqueous solution. Though, the nature of the crosslink dominant in different tissues decides the particular solvent to be used and the resulting yields [6].

Dermal layer equivalent (DLE)

It is an *invitro* model of the dermal layer of skin it is constructed by seeding dermal fibroblasts into a collagen gel. This gel may then be allowed to contract as a model of wound contraction. This collagen gel contraction assay may be used to screen for treatments which promote or inhibit contraction and thus affect the development of a scar. Other cell types may be incorporated into a dermal equivalent to increase the complexity of the model. For example, keratinocytes may be seeded on the surface to create a skin equivalent, on macrophage may be incorporated to model the inflammatory phase of wound healing [7].

Dermal skin replacements add greater mechanical stability and prevent the wound from contracting. Transcyte, a product made by Advanced Tissue Sciences, Inc. (La Jolla, CA, USA), utilizes seeded neonatal human dermal fibroblasts in a polymeric scaffold that is then cryopreserved, making it a non-living wound covering. Transcyte has been successfully used as a temporary wound covering after the burn wound has been excised. A derivative of this product, Derma graft by advanced biohealing, utilizes a biodegradable polygalactin mesh and has shown limited success in diabetic foot ulcer treatment Life cell (Branchburg, NJ, USA) developed alloderm and Strattice, intact a cellular matrix produced from cadaver skin by removing epidermis and the antigenic cellular elements in the dermis. Often, autologous keratinocytes were seeded and cultured on alloderm to form epithelium, and the epithelium-Alloderm structure can be applied for wound and burn closure [19]. A composite skin graft composed of an outer layer of thin silicone film and an inner layer Constructed of a complex matrix of cross linked fibres is marketed under the product name Integra Dermal Regeneration Template (Intergra DRT, Integra Life Sciences Corp.; Plainsboro, NJ, USA). Once dermal layer is regenerated, the silicone film on the dermal layer can be removed and replaced with an epidermal auto graft. Integra DRT has been successfully shown to treat burns [19]. The present study aim is fabricate the layer of sandwich composite using biopolymer and antibacterial agent; biological characterization of the developed

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dermal layer equivalent (DLE).

II. MATERIALS AND METHODS

A. Material and chemicals used

Fish scales were collected from nearby fish market. Cleaned and rinsed twice in water to remove dirt and extra flesh and kept in sun for drying. Neem was selected as Herbal drug for loading. 80% ethanol was taken and filtered, further condensed for research purpose.

S. No	Chemical	Make
1	polyvinyl alcohol (PVA)	Rankem
2	Glutaraldehyde (GA)	
3	Silver nitrate	

B. Test bacteria

Staphylococcus aureus (ATCC 6538) and *Escherichia coli* (ATCC 11230) were used as standard Gram positive and Gram-negative organisms respectively.

C. Preparation of fish scale collagen

The isolation of collagen from fish scales was performed using a modified method reported by [13]. In brief; fish scales were washed with running water to remove sand and other foreign bodies and later exposed under sunlight. 200g of fish scales were soaked in 10% sulphuric acid solution for 24h. The fish scales were then minced with an industrial mixer. The resultant fine paste was subjected to centrifugation (12,000rpm) at 4°C for 20mins. The supernatant was collected and its pH was adjusted to 7 using hydroxide solution. The collagen was collected from the supernatant solution (60% solids) and was stored at 4°C for further use.

D. Preparation of fish scale collagen film

50ml of fish scale collagen (1% wt. / wt. in water) and 50ml of PVA solution (1%wt. /wt. in water) were mixed in 250ml beaker and stirred for 1h at 60°C to obtain homogeneous solution. To this solution 1ml of 2% glutaraldehyde solution was added under stirring at room temperature (25°C). The solution was transferred immediately into a Teflon sheet covered glass plate (dimension: 110 mm length × 100 mm width × 3 mm height) and dried at 80°C in an electric oven for 2h. The formed films were washed with double distilled water to neutralisation and dried at room temperature. Similarly, 50 ml of 2% wt./wt. Collagen solution + 50ml of 1% wt./w t. PVA solution (2:1) and 2ml of 2%wt./wt. GA (Glutaraldehyde).

E. Preparation of chitosan-PVA silver nanoparticles films

Silver nitrate (100mg) was added separately into three beakers containing 50 ml of different percentages (1%, 2% and 3%) of chitosan solutions at room temperature. The corresponding solutions were kept in sunlight for 1h. The yellow coloured solution started turning to red, then brown and brownish indicating the formation of silver nanoparticles. To this AgNP solution, 50ml of 1%PVA solution was added and stirrer for 1h. To all these solution, 1 ml of 2% glutaraldehyde (cross-linker) was added under stirring at room temperature. The solutions were then poured into Teflon covered glass plates and dried as explained earlier. These films were termed as chitosan-PVA silver nanoparticles (CPSNPs) films.

F. Preparation of Oligochitosan film

50ml of Oligochitosan (1%wt. /wt. in water) and 50ml of PVA solution (1%wt. /wt. in water) are mixed in 250ml beaker and stirred for 1h at 60°C to obtain homogeneous solution. To this solution 1ml of 2% glutaraldehyde solution in water is added under stirring at room temperature (25°C). The solution is transferred immediately into a Teflon sheet covered glass plate (Dimension: 110 mm length × 100 mm width × 3 mm height) and dried at 80°C in an electric oven for 2h. The formed films are washed with double distilled water to neutralisation and dried at room temperature.

G. Herbal extraction

6 g of the powder plant which have medicine values in 100ml of 80% methanol are mixed in beaker and kept overnight under shaking condition. The solution was filtered by using whatmann No.1 filter paper and collect filtrate. The filtrate

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was evaporated at room temperature for complete evaporation of solvent and condensation of extract for 24 hours later stored at 4ºC.

H. Herbal capsule preparation

75ml of calcium chloride solution was added in drop wise to 125ml of sodium alginate under constant stirring (1500rpm) for 30min at room temperature. Herbal extract was added to the mixture by drops in 45-60min, after it was kept for overnight without any disturbance. The uppermost layer was discarded and the pellet was collected carefully.

I. Loading of herbal drug

For loading Herbal, the solutions (50mg) were allowed to swell in 20ml of Herbal solution (5mg of Herbal powder in 20ml of buffer containing acetone (8ml) -distilled water (12ml) for 24hrs at 25ºC. To this solution 1ml of 2% glutaraldehyde solution in water is added under stirring at room temperature (25ºC). The solution is transferred immediately into a Teflon sheet covered glass plate (Dimension: 110 mm length × 100 mm width × 3 mm height) and dried at 80ºC in an electric oven for 2h. The formed films are washed with double distilled water to neutralisation and dried at room temperature.

J. Antibacterial activity against MRSA

Methicillin-Resistant *Staphylococcus Aureus* (MRSA) infection is caused by a strain of staph bacteria that's become resistant to the antibiotics commonly used to treat ordinary staph infections. The antibacterial activity of the three composites was evaluated by MRSA method. Sterile Nutrient agar plates were prepared. 0.1ml inoculum of *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 8739) were swabbed uniformly over the surface of the agar. The sample film to be tested was placed at the centre of the plate and the plates were kept for incubation at 37ºC for 24 hours. The antibacterial activity was evaluated in terms of zone of inhibition (millimetres).

III.EXPERIMENTAL RESULT

Since its discovery approximately 200 years ago, chitosan, as a cationic natural polymer, has been widely used as a topical dressing in wound management owing to its haemostatic, stimulation of healing, antimicrobial, nontoxic, biocompatible and biodegradable properties. Chitosan interacts very easily with bacterium and binds to DNA, glycosaminoglycans and most of the proteins thereby enhancing the antimicrobial effect of silver nanoparticles. Fish scales are bio composites of highly ordered type I collagen fibres. Fish scale collagen was prepared by demineralization. Because of demineralization, the plywood-like structures were exposed thus making it easier to dissolve collagen by acid solution. Thin films by solvent evaporation from collagen extracted from fish scales were prepared.

Chitosan stabilized silver nanoparticles form a chitosan-PVA silver nanoparticles films where glutaraldehyde act as a cross linker. The reduction of silver ions into silver nanoparticles (AgNPs) is achieved in acidic solution of chitosan and poly vinyl alcohol (PVA) using their functional groups (-OH, -COOH, -NH2 groups).Chitosan forms viscous solution in various organic acids. These viscous solutions have been used to make functional films. The anti-bacterial activity of the chitosan-PVA silver nanoparticle film has demonstrated significant effects against *E. coli and S. aureus*. The plates were examined for evidence of zone of inhibition, which appear as a clear area around the film and the measurement was found to be 22mm. The results prove that the silver nanoparticles incorporated composite film has a better antibacterial property, thus may be potentially applied to a broader field in skin repair such as full thickness defect and burn. It is also used in biological characterization of the developed dermal layer equivalent.

IV. DISCUSSION

A Trilayer Dermal Equivalent (TDE) with enhanced antibacterial property was fabricated by incorporating silver nanoparticles. Composites are multiphase systems in which the macroscopic properties are dominated by interfacial interactions. These materials have recently become particularly interesting because of dispersed phase formed by metallic or metallic oxides nanoparticles in a matrix which can be amorphous or crystalline. During the processes of fish, a great amount of fish scales are dumped, which is a great waste because the scales of carp fish contain a large amount of collagen. Therefore in this paper, extraction and partial characterization of collagens from fish scale was potential utilized. Chitin or poly (β (1-4)-N-acetyl-D-glucosamine) is one of the most abundant polysaccharides found in nature. It can be found in skeletal materials of crustaceans, cuticles of insects, and cell walls of various fungi. Chitosan is prepared by chemical N - deacetylation of chitin. Both of them are observed to have biological functions. The cationic properties of chitosan offer the film-maker an additional opportunity to take advantage of electrostatic interactions with other anionic polysaccharides. These films were high modulus, flexible self-supporting and

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biodegradable [20]. Chitosan is non-toxic, biodegradable and biocompatible polymer. Chitosan widely existing in the nature and has antibacterial effect, heavy metal adsorption effect, antioxidation effect and film formability. Antibiotic therapy in recent years has faced difficulties due to the rapid emergence of multidrug resistance among bacteria causing several life threatening infections, and this in turn, making the future management of infectious diseases uncertain [21]. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for potential antimicrobial activity, and the plant extracts were found to have potential against microorganisms [22]. A dermal equivalent having a trilayered structure was designed by combining a silver nanoparticles incorporated chitosan film with a bilayer collagen-chitosan. The dermal equivalents, which possess the ability to induce in situ cell infiltration and regeneration of the damaged dermal layer such as Integra, are more attractive due to their lower production and management fees. Traditional split-thickness skin grafts for the resurfacing of large burns are the gold standard and provide permanent wound closure. Indeed, most of the full thickness burn wounds are satisfactorily closed as quickly as possible with the split thickness auto-graft [23].

V. CONCULSION

In conclusion, the trilayer dermal equivalent can be served as a more promising dermal equivalent for uses in broader types of skin defects.

Fig.1: Fish scales

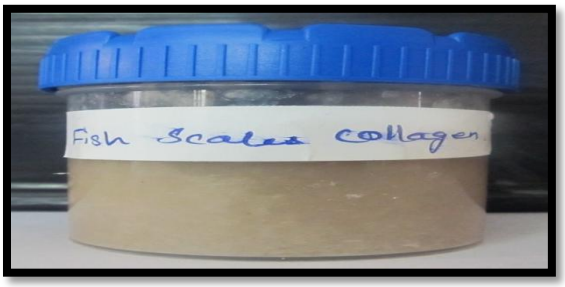

Fig.2: Fish scales collagen

Fig.3: Silver nanoparticles

Fig.4: Collagen silver

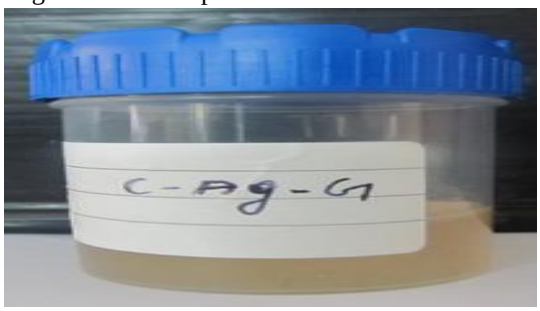

Fig.5: Collagen silver – GMA

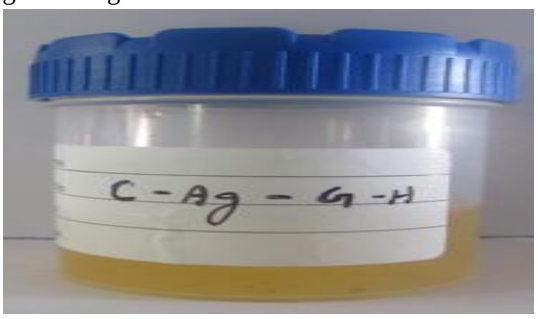

Fig.6: Collagen silver nanorods

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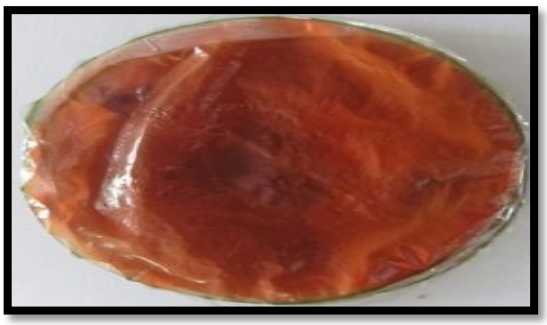
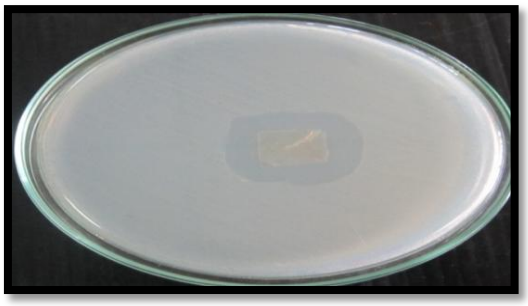


Fig.7: Biopolymer Films

(Fig. 8a) Herbal capsule film

(Fig. 8b) Chitosan film

(Fig. 8c) Collagen film



(Fig. 8d) AgNRs (Silver np) film

Fig. 8: antibacterial activity layer by layer sandwich composite against MRSA

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