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Antimicrobial and biocompatible fluorescent hydroxyapatite-chitosan nanocomposite films for biomedical applications

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ABSTRACT

Development of fluorescent erbium doped hydroxyapatite (eHAp)-chitosan nanocomposite film is reported. Nanocrystalline eHAp has been synthesized by hydrothermal assisted precipitation method using erbium (III) ions as dopant. Physico-chemical characterization by UV/Visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), photoluminiscence spectroscopy (PL) and Field emission scanning electron microscopy(FESEM) confirmed incorporation and uniform distribution of eHAp in the chitosan films. Strong antimicrobial activity was observed using eHAp incorporated chitosan films against *E. coli* and *S. aureus* by contact inhibition on agar plates. On the other hand, excellent biocompatibility was observed with human lung fibroblast cells (WI-38) which showed strong attachment and proliferation on the chitosan films with minimal cytotoxicity. Moreover, the doped films showed good biodegradation and mineralization behavior after 2 weeks in simulated body fluid. Thus the doped fluorescent chitosan films with multifunctional attributes can be a strong candidate for diverse applications like in antimicrobial treatments, wound healing, tissue scaffolds and bioimaging.

1. Introduction

Chitosan based films and nanocomposites have receiv-ed great attention in recent times for its desirable antimicrobial, biodegradable and biocompatible attributes combined with natural abundance and economical processing. Chitosan is derived from the deacetylation of chitin (which is naturally found in the exoskeleton of crustaceans) and is made up of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic linkages. Due to its microbicidal and nontoxic nature, chitosan has found diverse range of usage such as in food packaging and preservation which includes coatings on bread, fruit, vegetable, eggs, and various meat products and in healthcare and biomedical field such as in drug delivery, wound dressing material, skin grafts and tissue regeneration scaffold [1–5]. The positively charged amino group is instrumental in interacting with biological membranes and makes it a potent antimicrobial moiety. However, antimicrobial property of chitosan is also depended on several factors such as degree of acetylation, pH, type of microorganism, cell age, presence or absence of metal cations, pKa and molecular weight [2,6]. For example, Kong *et al* investigated physico-chemical factors that influence the antimicrobial property of chitosan [7]. In another study, Escárcega-Galaz et al reported on the antimicrobial activity of chitosan based films against *Salmonella typhimurium*and *Staphylococcus aureus* [8]. While Rhazi *et al* illustrated the effect of different chitosan sources and deacetylation process on the physiochemical characteristics of chitosan [9]. Li *et al* explained the antimicrobial mechanism of chitosan based on molecular weight with low molecular weight chitosan showing high antimicrobial activity due to higher percentage of amino group protonation and cation formation [10]. Jayakumar et al demonstrated that Chitosan based antimicrobial films can be effectively used to treat nosocomial infections arising from Streptococcus, Staphylococcus and Pseudomonas species during surgery and is also an excellent wound or burn dressing

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material on its own due to its adhesive nature,biocompatibilityand permeability to oxygen [11].

Apart from antimicrobial applications, chitosan based nanocomposites have been widely used as functional biomaterials ranging from drug delivery to tissue regeneration systems that interact with mammalian cells to influence their growth in a positive way. Generally, these nanocomposites consist of various bioactive molecules like drugs, enzymes, genes, nanoparticles embedded in the chitosan matrix [1,3–5]. One of the most commonly used biomaterial is the hydroxyapatite incorporated chitosan for bone tissue engineering applications. Due to closer resemblance to the chemical and mineral components of natural bone and excellent osteoconductive behavior, synthetic hydroxyapatite is widely used for hard tissue repairing, bioceramic coating, and dental applications [12,13]. Thus combining chitosan and HAp into a hybrid composite will showexcellent potential as bioactive material with enhanced mechanical, chemical and biological properties [14,15]. Recently, luminescent biomaterials based on hydroxyapatite have garnered wide interest and are widely used as fluorescent probe to study cellular membranes, imaging tissues and intracellular structures and drug delivery applications. For example, fluorescent rare earth doped HAps were investigated by several research groups to be excellent agents for bioimaging applications [16–21]. Similarly, Li et al developed fluorescein isothiocyanate functionalized HAp nanoparticles for cell labelling, animal imaging, and drug delivery applications [22]. Thus combining a fluorescent bioactive material such as hydroxyapatite with chitosan will be an ideal choice toward developing an advanced biomaterial suitable for not only therapeutic applications but also imaging cell-material interaction.

In the present work, we used erbium ion doped hydroxyapatite (eHAp) to develop luminescent chitosan-hydroxyapatite nanocomposite films which can be used both as an antimicrobial dressing material and a fluorescent cell regeneration scaffold or implant material. The developed chitosan films are flexible, easy to process and detailed characterization by various analytical techniques and biochemical assays indicated excellent antimicrobial, bioactive and fluorescent properties under *in vitro* conditions.

2. Experimental

2.1. Synthesisof erbium doped hydroxyapatite(eHAp) powder

Initially, in 40 mL of 0.03(M) calcium chloride (Merck, India) solution, 0.662 g erbium (III) chloride hexahydrate ($ErCl_3.6H_2O$, Sigma-Aldrich) was dissolved by stirring at room temperature. Next, 30 mL of diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Merck, India) was prepared and pH of the solution was adjusted to 10.5 by dropwise addition of 1 (M) sodium hydroxide (NaOH, Ranbaxy laboratories limited, India). The first solution was then added drop wise to the second solution by stirring and after 30 min, the total solution mixture was transferred into a teflon lined hydrothermal autoclave for 6 h at 140 °C. The resultant hydrothermally treated precipitate was washed several times by centrifugation to remove excess NaOH. The pale yellow precipitate was then dried at 60 °C to obtain erbium dopedhydroxyapatite powder. The mole ratio of Ca/P and Ca/erbium was 1.66 and 1.5 respectively [17].

2.2. Synthesis of eHAp doped chitosan film

Three different concentrations (1, 1.5 and 2.5% w/v) of chitosan (Mw: 100,000-300,000, DA- 85%, Acros-Organics, USA) films were prepared each containing 25% of eHAp by weight (Fig. 1). Briefly, calculated amount of chitosan and eHAp powders were dissolved in 1% acetic acid solution and stirred overnight at 60 °C. The solutions were then poured into plastic petri dishes and dried at 60 °C. The resultant films were collected and stored for further investigations [15,16]. Henceforth, the films will be designated as 1CH, 1.5CH and 2.5CH respectively.

2.3. Materials and methods

CH films were characterized by a Bruker AXS (Model D8, WI, USA) model X-ray Diffractometer (XRD) using CuKα radiation (1.5409 Å) and scan range (2θ) from 0 to 70° (at 40 kV voltage). Absorption spectra of the films were measured by UV/Visible spectrophotometer (UV 1800, Shimadzu) in the 200–800 nm wavelength range. The interaction between chitosan and eHAp was studied by fourier transform infrared spectrometry (FT-IR, FTIR-8400S model Shimadzu, Tokyo)with thescanning range set from 400 to 4000 cm^{-1} under Happ–Genzel configuration and a resolution of 4 cm^{-1}. The photoluminescence spectra of 1 mg/mL eHAp solution in absolute ethanol medium was investigated using Cary Eclipse fluorescence spectrophotometer, Agilent Technologies and microscopic images of eHAp powder and eHAp doped CH films were taken by Zeiss Axiocam MRc fluorescence microscope. Microstructure of the CH films was observed under field emission scanning electron microscope (FESEM, model FEI Quanta 50 (USA). A portion of the films were cut and gold coated *via* plasma spraying at 0.1 mbar pressure. The emission current and operating voltage were kept at 170 μA and 20KV, respectively.

Antimicrobial activity of CH films was studied by agar-diffusion method [9] and plate count method [23]. For agar-diffusion method, 20 ml nutrient broth (0.5% peptone, 0.1% beef extract, 0.2% yeast extract, 0.5% NaCl, (Himedia Pvt. Ltd., India)) containing 20%agar was taken into sterile petri dishes and allowed to solidify. Next, 100 μL of overnight grown microbial culture (cell density- 10^7 CFU/mL) was dispersed over the medium by sterile swab. The films were then placed carefully on the solid media to ensure direct contact with the film surface. The plates were then incubated at 37 °C for 24 h. Direct contact between film and medium surface is a crucial factor to assess the bactericidal effect of chitosan membranes [9,24]. For plate counting method, microbial culture with same cell density was incubated for 24 h in nutrient broth (composition as described above but without agar) containing three different CH films. After incubation, an appropriate inoculum was plated on nutrient agar plates. Mortality rate was calculated by counting the number of colonies on control and treated plate after 24 [23].

The morphological characteristics of the microbial cells after interaction with the CH films were observed by FESEM. This procedure was done on the suspensions of treated cultures after 12 h of incubation. Briefly, mid exponential phase bacterial culture(final cell density ~ 10^7 CFU/mL) were incubated with 1CH, 1.5CH and 2.5CH films at 37 °Cfor 24 h. Control (without the films) was prepared under same conditions. After treatment, the cells were collected by centrifugation at 5000 rpm for 10 min followed by repeated washings with phosphate buffer saline (PBS) and fixation with 2% formaldehyde (37–41% w/v, Merck, India) (in PBS). Samples were then dehydrated by serial dilutions of ethanol and 5 μL was drop casted on a clean glass cover slip. After drying, the samples were sputter coated with gold and observed under electron microscope [23,25]. Qualitative determination of hydrophillicity of CH film was performed by placing a single drop of distilled water on the film and taking a cross-sectional image using DSLR camera (Nikon D3300).

Cytotoxic effect of CH filmson human lung fibroblast (WI-38) cells was determined by Methyl tetrazolium based (MTT, Biologos Inc., USA) cell viability assay.Briefly, sterile chitosan films were incubated with human lung fibroblast (WI-38) cells (cell density 5×10^3per well in 96 well plate) in Dulbecco Modified Eagle Medium (DMEM) medium, (Biologos Inc. USA) for 24 h. Thereafter, the cell suspension was treated with 5 μL of 15 mg/mL MTT solution followed by 4 h incubation at 37 °C. The MTT solution was then removed and 100 μL of DMSO was added to dissolve the formazan crystals and generate homogeneous purple solution. Cytotoxicity was determined by measuring absorbance at 570 nm using a microplate reader. By comparing this absorbance