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Synthesis and characterization of human bone-like hydroxyapatite using Schiff's base

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ABSTRACT

Hydroxyapatite (HAp) has prospective applications in the field of biomaterials as it has good biocompatibility and mechanical reliability. HAp has been synthesized using surfactants and organic modifiers, which follow the mechanism of chelating the calcium and phosphate precursors to form the calcium phosphate mineral mimicking the mineral in native human bone. The method followed in this work exploits schiff's base which follows the same mechanism of chelating the reactants necessary for HAp formation. Needle-like nanostructures were formed in the presence of the schiff's base derived from salicylaldehyde and 1, 4-diaminobutane. The synthesized schiff's base and HAp were characterized using FT-NMR, XRD and FT-IR to confirm their chemical structure and crystallinity. The cytotoxic evaluation of the synthesized HAp was performed using MTT assay with murine fibroblast (L929) cells, which proved the proliferation activity of the cells in presence of HAp was found to be directly proportional to the time of exposure and HAp concentration, hence, inferring that the synthesized HAp is not only biocompatible but also promotes cell proliferation.

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the most important constituent of bone tissues and teeth. Hydroxyapatite (HAp) is present as the mineral component of the naturally occurring bone. HAp present in human bone is present in the form of apatite platelets of 15–200 nm length, 10–80 nm width and 2–7 nm thickness [1,2]. However, there is always a debate about the morphology of the HAp minerals present in the bone, whether they are needle-shaped and platelet-shaped mineral particles. Native-HAp compared to other calcium phosphate salts was found to have enhanced bone remodeling and ingrowth, which shows that they have better osseo-integration and osseo-conduction properties [3,4]. Hence, HAp synthesized for biomedical applications have to accommodate properties such as osseo-conduction, osseo-integration and osseo-induction in vivo for the scaffold/implant to mimic the native bone tissue.

There are numerous synthesis methods of HAp which has been already studied and experimented in detail in literature. The methods include conventional synthesis techniques with chemical approach such as wet chemical precipitation method where a precipitate is obtained from the reaction of precursors to obtain HAp of nano-plate like morphology [5,6]; solid-state reaction method where the precursors are sintered at high temperatures to obtain micro and nano-particles [7,8]; hydrothermal method where the precursors are allowed to react in an aqueous solution with high temperature and pressure influence to obtain HAp crystals [9,10]; sol-gel method where precursors are not treated at high temperatures until a gel-like network is formed to obtain globular-shaped HAp nanoparticles [4,11], mechanical approach such as mechanochemical method which involves coupling of mechanical forces and chemical reactions to obtain nanorods and/or nanofibres [12,13], deposition approach such as biomimetic deposition method where the precursors are added to synthetically prepared simulated body fluid and ultrasonically irradiated to obtain micro-sized HAp particles [14,15], and electrodeposition method where electric current is used to form nano-sized HAp coating on an electrode [16,17]; complexing approach where polymers such as polyethylene glycol is added along with the precursor solutions and reaction is carried out as similar to wet chemical precipitation to obtain HAp nanoparticles [18–20]; and other miscellaneous methods such as emulsion method where aqueous solvent containing the precursors are mixed with organic solvent containing surfactants to obtain HAp nanospheres [21,22], and ultrasonic spray pyrolysis method where precursor solution is directed through ultrasonic nozzle on a substrate to produce HAp nano-rods [23,24].

A novel approach inline to the complexing agents has been brought to light by exploiting schiff's bases as chelator/complexing agent/organic modifier to synthesize HAp [25–27]. Schiff's base is a product obtained from reaction with a primary amine and an aldehyde/ketone, which can be used as a chelator to bring calcium and phosphate

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precursors in proximity for HAp formation. This work which was not much explored in literature follows the same principle as explained above which exploits schiff's base synthesized from salicylaldehyde and 1,4-diaminobutane. Also, there can be several toxicity issues arising from using such organic entities in the synthesis process of HAp. Hence, it is recommended to analyze the cytotoxicity of the synthesized HAp prior to its use in fabrication of biomaterials. This study also gives adequate evidence that HAp synthesized by the schiff's base method is not cytotoxic. The chemical structure of schiff's base was characterized using ^{1}H and ^{13}C NMR, and FT-IR. The prepared schiff's base was then used for the preparation of HAp. HAp was characterized using FT-IR, XRD and EDS for confirmation of chemical structure, SEM and TEM for morphology and size, and MTT assay for cytotoxic analysis.

2. Materials and methods

2.1. Materials

Salicylaldehyde was purchased from Himedia Laboratories (Mumbai, India); 1, 4-diaminobutane was purchased from Sigma-Aldrich (Bengaluru, India). Methanol, calcium nitrate tetrahydrate, potassium dihydrogen orthophosphate and chloroform were purchased from S D Fine-Chem Limited, Mumbai, India.

L929 cell lines were obtained from NCCS, Pune. DMEM, FBS, trypsin and 1X antibiotic solution were purchased from Hi Media Laboratories, Mumbai, India. Methylthiazolyl diphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemicals Company, Mumbai, India. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (10,000 U/ml), and streptomycin (10 mg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

2.2. Synthesis Of Schiff's Base

Schiff's base was synthesized by reacting salicylaldehyde (2 mM, 0.243 g) and 1, 4-diaminobutane (1 mM, 0.088 g) in methanol (10 ml). The mixture then kept into a microwave oven for reaction to occur was maintained at T = 70 °C and power of 350 W for 5 min. The resulting mixture was allowed to cool and filtered using membrane filter paper (Axiva membrane filters with pore size of 0.45 µm). The residue was then recrystallized using heated methanol (at approximately 40–50 °C) and filtered. The filtered residue was dried in open air at room temperature overnight.

2.3. Synthesis Of hydroxyapatite nano-needles

The synthesized schiff's base (0.0944 g) was dissolved in methanol (40 ml) under constant stirring for 5 h. Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) (10 mmol, 0.0944 g) was added to the dissolved schiff's base-methanol solution, and was allowed to thoroughly mix for another 30 min. Phosphate precursor solution was prepared by dissolving potassium dihydrogen orthophosphate ($(NH4)_2HPO_4$) (6 mmol, 0.0326 g) in methanol (40 ml). To assure monodisperse and uniformly shaped HAp nanostructures, drop wise addition of urea (NH_2-CO-NH_2) (0.1 M) to phosphate precursor solution was performed until the pH was adjusted to 12. This solution was slowly added to the calcium-Schiff base solution at the rate of 5 ml/min to obtain a milky suspension. This mixture was heated in an oil bath at 120 °C for 5 h. The precipitate was collected by centrifugation at 5000 rpm for 30 min after cooling the reaction mixture, and washed sequentially with water, methanol and chloroform. The obtained precipitates were dried for 5 h in hot air oven at 50 °C.

2.4. Characterization

Fourier transform infrared (FT-IR spectrophotometer - SHIMADZU CROP IRAFFINITY-1) spectra were acquired in the range of 4000–400 cm^{-1} for schiff's base and HAp samples. The spectra were obtained with 30 scans per sample. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were acquired for schiff's base on a BRUKER (400 MHz) spectrometer. Powder X-ray diffraction (BRUKER Germany with Cu K radiation; = 1.5405 Å) patterns were collected for HAp in the 2θ range of 10–80°. Scanning electron micrographs (ZISS-EVO18) were taken for HAp at a voltage of 15 kV. Transmission electron images (HR-TEM - FEI-Tecnai G2 20 S-TWIN High resolution) were recorded for HAp at a voltage of 200 kV. Energy dispersive spectrometry (EDS) analyses were carried out on Bruker QUANTAX for HAp along with HR-TEM micrography. The synthesized HAp was also studied using thermal gravimetric analysis (TGA- SDT Q600 V20.9 Build 20) and differential scanning calorimetry (DSC- Shimadzu DSC-50 system) for its properties under the influence of higher temperatures upto 1200 °C at a heating rate of 20°C per minute.

2.5. Cytotoxicity assay

When synthesizing or fabricating any material in relation to biomedical application, it is significant to check the biocompatibility of the material against live cells, which was carried out as explained below. Biocompatibility of synthesized HAp was evaluated using murine fibroblast cells (L929). Cells were grown in DMEM supplemented with 10% FBS and 1X penicillin/streptomycin antibiotic solution. The cells were maintained in a humidified incubator (ESCO Cellmate Biotech) at 37 °C with 5% CO2 environment. HAp powder was sterilized by washing twice with double distilled water, twice with ethanol, and once with sterilized phosphate buffered saline (PBS) sequentially and left for ultra-violet light exposure overnight (approximately for 12 h). Before cell seeding, sterilized hydroxyapatite of 4 different concentrations (1.4, 2.8, 4.2 and 5.6 mg/ml) was dispersed and sonicated using a bath sonicator (Citizon – CUB 2.5 l) for 20 min respectively. After culturing the cells in the presence of hydroxyapatite (sonicated extract) for 1 day, 2 days and 7 days, the existent medium was removed and MTT (20 µl, 1 mg/ml) was added to the cultures respectively. Cells were incubated at 37 °C in humidified atmosphere (5% CO2) for 4 h. After incubation, 100 µl of DMSO was added to each well to dissolve purple crystals of formazan. The absorbance was measured in a spectrophotometer (readwell TOUCH, ROBONIK) at a wavelength of 540 nm. To eliminate the absorbance obtained from HAp, the supernatant obtained from centrifugation of formazan-MTT_DMSO solution is measured for absorbance. Reported values are the means of three experiment groups with 3 replicates each (5% significance level) and are expressed as percentages of the control values.

The % cell viability was calculated using the following formula:

$$\%\quad \textbf{Cell Viability} = \frac{A_{570}\ \textbf{of}\ \textbf{treated Cells}}{A_{570}\ \textbf{of}\ \textbf{control cells}} \times 100$$

2.6. Statistical analysis

Data were expressed as mean ± standard deviation of 3 replicates for each experiment. Each experiment was repeated at least thrice. Statistical comparisons were made between different concentrations and different time intervals using 2 way ANOVA, and p value of 0.05 was considered to be significant.

3. Results

3.1. NMR and FT-IR Studies of Schiff's base

Chemical structure of the synthesized schiff's base was studied by ^{1}H NMR and ^{13}C NMR and the respective spectra are represented in Fig. 1a and b. The characteristic details of the NMR spectra of the schiff's base is as follows: ^{1}HNMR (400 MHz, $CDCl_3$): δ = 1.812 (4H,