SYNTHESIS AND CHARACTERISATION OF GELATIN-PVA/HYDROXYAPETITE(HAP) COMPOSITE FOR MEDICAL APPLICATIONS

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Abstract. Biodegradable composite biomaterials play a pivotal role in the healthcare and addressing many challenging issues. Present study reports on the synthesis and characterization of hydroxyapatite (HAP) and gelatine-PVA composite, analyses the incorporation of HAP in gelatine-PVA and investigating their biocompatibility and mechanical properties. The material was prepared in film form by mixing freshly prepared HAP into gelatine-PVA solution and finally drying them to make the composite film. Composite gelatine-PVA were characterized by scanning electron microscopy (SEM), X-ray diffractometry (XRD), Fourier Transform Infrared (FTIR), hydrophilic-hydrophobic nature and evaluated for its strength, hemocompatibility and Cytotoxicity. This composite material as fabricated and characterized, may be used as a target material for bone refurbishment including dental filling patches.

Key words: Composite, Hydroxy apatite, Biocompatibility, Polyvinyl Alcohol
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1. INTRODUCTION

Biomaterials have great importance in the field of Biomedical Engineering. A biomaterial is any material, comprises whole or part of a living structure or biomedical device which performs, augments, or replaces a natural function. The material can be natural or synthetic and includes metals, ceramics and polymers. They mainly are used in the medical field for tissue repair, heart valves etc. In this present experiment, we have studied biomaterials that may be used in bone refurbishment.

Bone is a tissue that has the ability to heal and regenerate itself. Bone refurbishment is a surgical procedure that replaces missing bone in order to repair bone fractures that are extremely complex, pose a significant health risk to the patient, or fail to heal properly. Bone grafting is a surgical procedure that replaces missing bone in order to repair bone fractures that are extremely complex, pose a significant health risk to the patient, or fail to heal properly. Bone grafts may be autologous (bone harvested from the patient’s own body), allograft (cadaveric bone usually obtained from a bone bank), or synthetic (often made of hydroxyapatite or other naturally occurring and biocompatible substances) with similar mechanical properties to bone.

For more than 50 years, different materials, such as metals, ceramics, synthetic, and natural polymers have been used for bone repair with different physiological or mechanical properties.
characteristics. Manufacturing three dimensional porous scaffolds in recent years increased the hope for the production of scaffolds with closer similarities to bone matrix. Numerous biomaterials have been investigated as scaffolds for bone repair, including natural and synthetic materials. Amongst these, calcium phosphate-based materials, especially bioactive hydroxyapatite (HAP, \( \text{Ca}_6(\text{PO}_4)_2(\text{OH})_2 \)) are widely used because of their chemical and structural similarity to the mineral portion of bone [1, 2]. Natural bone is a three-dimensional composite made up of organic and mineral phases of which collagen is the main organic constituent and HAP is the main mineral one. To synthesize a scaffold that mimics the natural structure of bone, composite systems have been developed, which combine the desired properties of both organic and mineral phases into a single material system. The most widely investigated composites for preparation of tissue engineering scaffolds are polymer/ceramic systems, such as HAP/collagen [3], HAP/chitosan [4], HAP/collagen/poly(lactic acid) [5], HAP/alginate/collagen [6], and HAP/gelatine [7]. Gelatine (GEL), a derivative of collagen, is an attractive component for the purpose of extracellular matrix replacement as it contains several biologic functional groups, which enhance osteoblast adhesion and migration and mineralization. Polyvinyl alcohol has excellent film forming, emulsifying and adhesive properties. It is also resistant to oil, grease and solvents. It has high tensile strength and flexibility, as well as high oxygen and aroma barrier properties [8].

We have prepared a HAP-Gelatine-PVA film and performed different physical characterisation. We have also evaluated its biocompatible nature to find whether it could be found different medical applications.

2. MATERIALS AND METHODOLOGY

2.1. Chemicals.

HAP[\( \text{Ca}_6(\text{PO}_4)_2(\text{OH})_2 \)], PVA with a molecular weight of 115000 kDa, Gelatin (purified) and 2 % Glutaraldehyde Solution were purchased from Merck.

2.2. Preparation of Hydroxyapatite:

Hydroxyapatite was prepared by reacting calcium hydroxide(Ca(OH)\(_2\)) and phosphoric acid (H\(_3\)PO\(_4\)). When the reaction was over, the beaker was kept aside for 24 h. It was seen that a white powder like element has settled in the bottom of the beaker and the residual water had come up. The residual water on the top of the beaker was drained out with the help of a sucking tube, and the remaining white coloured product (produced due to the mixture of Calcium Hydroxide and Orthophosphoric acid) was filtered using vacuum pump.

The petridish filled with the end product of the reaction was then dried in an incubator at 70\(^\circ\)C. The dried samples were taken out from the incubator and calcinated in a furnace at 800\(^\circ\)C and heat flow inside the furnace was increased slowly with the help of the knob. It took around 2 h for the temperature of the furnace to reach 800\(^\circ\)C from normal room temperature. The calcination was done for 2 h. The samples were removed from the furnace after 24 h when the temperature slowed down.
2.3. HAP –Polymer Film Synthesis

200 mg HAP was taken in 10mL acetic acid solution and mixed thoroughly with 5mL of 5% Gelatin solution and 10 ml of 5% PVA solution and the solution was stirred in a Magnetic Stirring Plate (REMI) at a temp of 40°C –60°C for 30 minute. 1ml of 2% Glutaraldehyde was poured dropwise into the solution and the solution was again stirred for another 30mins at a speed of 650 rpm. The final solution was then solvent casted at 40°C for 24 h to obtain the desired membrane. A Gelatin-PVA film was also prepared in similar manner without the hydroxyapatite.

Fig1: (a) HAP preparation; (b) Filtering process; (c) Vacuum pump; (d) HAP after filtering; (e) Furnace machine for drying; (f) HAP after drying.

Fig2: PVA-Gelatine/HAP composite film
2.4. Characterization

**XRD:**
The phase analysis of the HAP powder and composite samples was done by XRD (Model PW 1729, Philips, Holland) using 35 milliamps, and 40 kV current. The mean crystallite size has been calculated using Scherer’s equation, that is, \( D = \frac{0.9 \lambda}{\beta \cos \theta} \), where \( D \) is the average crystallite size in A° , \( \beta \) is the peak broadening of the diffraction line measured at half of its maximum intensity in “radian,” \( \lambda \) is the wavelength of X-rays, and \( \theta \) is the Bragg’s diffraction angle[9].

**FTIR:**
The goal of FTIR is to find different functional group in the present sample [10]. The identification of functional groups in the HAP powder was analyzed by FTIR analysis on IR-Prestige-21 Shinad-ZU at 400-5000 range.

**SEM:**
Morphology of the films and pure HAP and PVA-Gelatine/HAP composite are observed by Scanning Electron Microscope(SEM) [11] in Inspect F50, model Q150R with 1400 (VA) power and 50/60 Hz frequency. The composite material was coated with gold before study to SEM.

**Contact Angle:**
Contact angle and biocompatibility is inversely related. When \( \theta=0 \) then it is highly hydrophilic and biocompatible and when \( \theta>90 \) it is hydrophobic. The surface property of glycerine and water is quite similar. The wetting property was analysed by measuring the contact angle of HAP-polymer composite film with respect to Glycerine.

**Mechanical Testing:**
Shore durometer is one of several methods used to find the hardness of a material. Higher numbers indicate harder materials; lower numbers indicate softer materials [12]. The Shore D hardness of the composite material was measured using a SHORE-D meter.

2.5. Biocompatibility test

**Hemocompatibility test:**
Hemocompatibility of the Composites has been estimated by Hemolysis Studies Fresh human blood, collected in a EDTA tube was diluted with normal saline solution (2 mL blood + 2.5mL normal saline). A standard sample without sharp edges was kept in a centrifuge tube containing 10 mL of normal saline was kept in an incubator at 37°C for 30 min. To this was added 0.2 mL of the diluted blood which was then mixed gently and incubated for 60 min. For the positive control, 0.2 mL of diluted blood was taken in 10 mL of 0.1% sodium carbonate solution and for negative control, 0.2 mL of diluted blood was taken in 10 mL of normal saline solution and incubated for 60 min at 37°C. In a similar way, sample material was incubated for 60 min at 37°C. After 60 min of incubation, all the test tubes were centrifuged for 5 min at 4000 rpm and the supernatant was carefully removed and transferred to the cuvette for readings at 545 nm wavelength and percentage hemolysis was calculated [14]. Percentage hemolysis is calculated using percentage equation (1) based on average of three replicates.
Percentage Hemolysis = \[\frac{(OD_{test} - OD_{negative})}{(OD_{positive} - OD_{negative})} \times 100 \]  

When the percentage of hemolysis is less than 5% then it is highly hemocompatible, when percentage hemolysis of sample within 10% then the sample is hemocompatible and when greater than 20% then the sample is non hemocompatible.

**Cell Viability Study:**
The viability test was done by the MTT assay. This colorimetric assay uses reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity for cell viability. Viable cells contain NAD (P) H-dependent oxidoreductase enzymes, which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple colour. Formazan crystals are then dissolved using a solubilizing solution and absorbance is measured at 595nm. The darker the solution, the greater the number of viable, metabolically active cells. Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation (see formula below). Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation.

\[\text{% viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100 \]  

\[\text{.......................... (2)}\]

### 3. RESULTS AND DISCUSSION

#### 3.1. Characterisation study

##### 3.1.1. XRD study

![Fig3: XRD curve of Pure HAP](image)

The diffractogram resulting from XRD analysis of the synthesized powder is shown in Fig3. The straight baseline and the sharp peaks crystal of this diffractogram in 211, 112, 300, 222...
and 213 was measured in $30^\circ - 60^\circ$ range which confirms that the product is well crystallized.

### 3.1.2. FTIR study

![FTIR Study](image1.png)

**Fig4:** (a) FTIR study of pure HAP (b) FTIR study of PVA-Gelatine/HAP composite

In Fig4 (a) FTIR Spectra clearly confirms the presence of $\text{PO}_4^{3-}$, $\text{OH}^-$ and $\text{CO}_3^-$ group in the developed products. The peak observed around 3630 cm$^{-1}$ is due to the presence of $\text{–OH}$ bond. This peak is mainly due to O-H stretching vibration in HAP. The peak at 1550 cm$^{-1}$ for $\text{CO}_3^-$ and the peak at 1100 cm$^{-1}$ is associated with the stretching modes of the P-O bonds of HAP. The double peak at around 600 cm$^{-1}$ is due to the bending modes of P-O bond in phosphate.

The interfacial interaction between PVA-Gelatine/HAP composites was confirmed by FT-IR spectra in Fig4 (b). The peak at 3650 indicates the presence of O-H stretching or this is majorly due presence of alcohol which is PVA in this case. Instead sharp a broad peak also indicative of H-bonded OH of PVA. Band at 1100 cm$^{-1}$ due to the stretching of the P-O bonds. The peaks at 1500 and 1600 could be attributed to the presence of aromatic C=C bonds stretching vibrations.

### 3.1.3. SEM:

![SEM Study](image2.png)

**Fig5:** SEM study of pure HAP in 5000 magnification
The analysis of SEM micrograph for HAP above shows in Fig5 fine agglomerates that are of irregular shape, oval and spherical-pellet-like shapes. Particle are formed from 0.4 μm to 1.6 μm in size.

Fig6: SEM study of PVA-Gelatin/ HAP composite in (a)1000 magnification; (b) 2500 magnification; (c) 5000 magnification and (d) 8000 magnification.

Fig6 shows the SEM micrographs of the composite sample in different magnification. Presence of HAP within the polymer matrix is clearly indicated through the SEM. As there is two type of polymer. Formation of different layer of polymer is also reflected in the SEM. Beside that presence spherical HAP particle size is around 0.9 μm to 0.14 μm which is nearly similar to that of only HAP as indicated in Fig5.
3.1.4. Contact Angle:

The contact angle of PVA-Gelatin/HAP composite film with respect to Glycerine measured 44° which is indicating the biocompatible property of the composite material.

3.1.5. Mechanical Testing

The hardness of the HAP-polymer composite film was tested using Shore D meter and the hardness of the film is 14 in the Shore D measurement scale which implies the composite material is very soft in nature.

3.2. Biocompatibility test

3.2.1. Hemocompatibility test

Table 1: Hemocompatibility Test (Human Blood)

| Sample                  | O.D at 545 nm | % Hemolysis | Remarks   |
|-------------------------|---------------|-------------|-----------|
| +ve Control             | 0.6629        | -           | -         |
| -ve Control             | 0.0038        | -           | -         |
| Gelatin-PVA /HAP composite | 0.0088      | 0.759%      | Hemocompatible |

Table 1 shows that the HAP-Polymer composite films are highly compatible with human blood.

3.2.2. Cell Viability Study

![Cell viability test](image)

**Fig7:** Cell viability test on HAP-Polymer composite film, PBS was taken as control.
It can be seen from Fig7 that the HAP-Polymer composite films are highly cell viable (above 90.14%) so these composite materials are highly biocompatible and has very less toxic effect.

4. CONCLUSIONS
In our work, we have seen XRD analyses clearly confirm the synthesized powder was nearly a pure HAP. All the typical absorption characteristics of HAP were also observed in FTIR analysis of the synthesized powder and the resulting scaffold. Hemolysis test and viability confirms that the developed composite is highly hemocompatible whereas contact angle property confirm its hydrophilic nature. From the hardness testing we knew this composite is very soft material and mtt assay confirms its biocompatible nature. So we can conclude it may be has application in vivo. Therefore, the developed Gelatine-PVA /HAP composite may have applications further evaluated in bone tissue engineering applications and in bone refurbishment.

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