Introduction

In view of the growing number of traffic accidents and human life expectancy, bone is emerged as the most frequently transplanted human tissue and its demand is growing in medical market for reconstruction of bone defects [1]. So researchers in the field of orthopedics have given considerable emphasis on the fabrication of reliable and economically feasible biomaterials for bone tissue replacement and regeneration applications. Current methods of broken and defective bone treatment use metal orthopedic implants, unfortunately shows many shortcomings such as insufficient prolonged bonding between the implanted material and juxtaposed bone, different mechanical properties between bone and the implant leading to stress shielding, release of certain ions and corrosion products from metallic implants and the need of second surgery to remove the implant [2].

Among the various bioceramics, hydroxyapatite (HA) is an ideal material to develop bone tissue engineering scaffold due to its osteoconductive and osteoinductive properties as well as the close structural composition to natural bone mineral. But its brittleness and poor performance in terms of mechanical stability limit its use for the regeneration of non-load-bearing bone defects. On the other hand, biocompatible polymers which are widely used in bone grafting also persists some practical problems such as low efficiency of cell seeding and poor mechanical property compared with natural hard tissue [3]. Since these currently designed scaffold materials fulfill only part of the requirements, it remains a great challenge for orthopedics researchers to design an ideal bone graft that emulates natures own structure.

Nanocomposites consist of bioactive polymers and ceramic nanoparticles are considered as the next generation scaffolds for tissue regeneration by overcoming the drawbacks of bioceramics and biopolymers [4]. Polysulfone is a transparent thermoplastic with physical properties matching to the light metals. Its chemical structure composed of phenylene units linked by three different chemical groups – isopropylidene, ether and sulfone-each contributes favorable properties such as high rigidity, creep resistance, bioinertness, and good thermal and chemical stability makes it a suitable candidate for bioactive nanocomposite [5]. Hydroxyapatite particles have been widely utilized in the fabrication of bone-like hybrid polymer nanocomposites due to its structural and compositional similarity to minerals of natural bones [6]. In particular, these nano-sized components in the polymer matrix provides multiple avenues to engineer implants by tailoring the surface for enhanced interaction with proteins, cells, and apatite nucleation at the same time creating implant materials with better mechanical properties.

Enlightened by this concept a novel tissue engineering scaffold in the form of thin film was developed with nano-hydroxyapatite (n-HA) and polysulfone using solution casting method. Since interfacial adhesion between organic polymers and inorganic n-HA, and the uniform dispersion of n-HA in the polymer matrix are the two important factors in fabricating nanocomposites, hydroxyapatite nanoparticles prepared by sol-gel method were organically modified with stearic acid. These modified hydroxyapatite nanoparticles were characterized by total reflectance fourier transform infrared spectroscopy (ATR-FTIR)), X-ray diffraction (XRD) and transmission electron microscopy techniques. The
morphology of the nanocomposites was studied by atomic force microscopy (AFM). The effect of addition of nanofiller on the hydrophobic and mechanical properties of polysulfone was examined. The bioactivity of the nanocomposites was evaluated by monitoring the concomitant formation of apatite on the material surface after soaking them in simulated body fluid (SBF). Moreover, the protein adsorption on the nanocomposite surface, which plays a prominent role in attachment and spreading of osteoblast cells, were studied qualitatively by AFM imaging and quantitatively by adsorption experiments.

**EXPERIMENTAL**

**Materials**

Commercial-grade Polysulfone Udel P-3500, used in this study was obtained from Amoco Polymers Inc., USA. Calcium nitrate tetra hydrate, trisodium phosphate, dimethyl formamide (DMF), Stearic acid, and ammonia (SRL, Mumbai, India), were used as received.

**Preparation of nano hydroxyapatite**

Calcium nitrate tetra hydrate and trisodium phosphate were used as calcium and phosphorus precursors for the synthesis of nano hydroxyapatite (HA) particles using sol–gel precipitation technique. 1 M calcium nitrate solution was added slowly to 0.6 M trisodium phosphate solution with stirring. To this mixture ammonia was added drop wise resulting in a white precipitates settling at the bottom of the beaker. The pH of the solution was kept 11 during the addition of ammonia. This precipitated solution was rigorously stirred for 2 h and aged at room temperature for 24 h. The precipitate was separated from the solution using filter paper and washed repeatedly using warm double distilled water. The precipitate cake obtained was dried at 60 °C for 24 h in a dry oven. This dried powder was calcined in air at 600 °C for 2 h using an electrical furnace and employing a heating rate of 10 °C/min. The prepared nano hydroxyapatite was modified with stearic acid to increase the interfacial adhesion between organic polymers and inorganic HA, and the uniform dispersion of HA at nano-level in the polymer matrix. [7].

**Preparation of nanocomposites**

PSf used was pre-dried in a vacuum oven at 120 °C for at least 12 h. A homogeneous solution of polysulfone is prepared by dissolving appropriate amount of polymer in DMF followed by stirring for 10 h at 50 °C. Then, stearic acid modified nanohydroxyapatite (n-SHA) was mixed with PSf solution and stirred at 50 °C for 24 h with varying filler amounts as 0, 1, 3 and 5 wt%. The solution was sonicated for 30 minutes to avoid agglomeration of n-SHA. Thin films of thickness 0.2 ±0.02 mm were prepared by solution casting method. The samples were air-dried under flowing air for 24 h to allow the solvent to evaporate and subsequently vacuum-dried at 40 °C for 48 h to remove any trapped solvent in the film. The PSf/ n-SHA composites were designated as PS0, PS1, PS3, and PS5 respectively according to the weight percentage of nanoparticle loading.

**Characterizations**

X-ray diffraction (XRD) patterns of n-HA, n-SHA, pristine PSf and nanocomposites were taken by a Bruker AXS D8 Advance diffractometer with Cu Kα radiation (λ=1.5406 Å). The samples were scanned with a step size of 0.02° in a range of 2θ from 0 to 70°. The operating voltage 40 kV and the current 30 mA of the tube were kept same throughout the investigation. Transmission electron microscope (TEM) (JEOL JEM-1011) was used to observe the morphology and the particle size of the powders. ATR-FTIR of samples was done using IFS 66 V/S (Bruker) spectrometer to study the bonding configurations of the samples. Scans were done in transmission mode from 4000 to 400 cm⁻¹. AFM images provide detailed information of the surface morphology of the nanocomposite films and helpful to investigate the interfacial properties. Tapping mode AFM images were recorded in ambient atmosphere at room temperature with Nanoscope III (Digital Instruments, Santa Barbara, USA). The probes were commercially available silicon tips with a spring constant of 20-80 N/m, a resonance frequency lying in the 255-300 kHz range. Images were analyzed using a Nanoscope image processing software.
Mechanical Testing

The tensile properties were investigated using Universal testing machine (UTM, H10KS Tinius Olsen, U.K) at a cross head speed of 10 mm/min with gauge length of 25 mm. Tests were carried out at room temperature using rectangular samples of 150 x 25 x 0.1 mm$^3$ (ASTM D 638). From the stress - strain curves the tensile strength, Young’s modulus and elongation at break was determined. At least five specimens were tested for each set of samples, and the mean values were reported.

Contact angle studies

Contact angle measurements were carried out by a Goniometer (GBX, France) using sessile drop method. The samples were dried at 60°C in a vacuum oven for 24 h prior to measurement. A MilliQ grade water drop of 5µL was slowly and steadily placed on a sample size of 100×100 mm$^2$, with a micro syringe. The contact angle was measured in air at room temperature within 30 seconds of the addition of water drop and photographs were taken. The average contact angle from at least six different locations on each polymer film was determined and the experimental uncertainty was within ±1°. The contact angle of water in air on the surface of nanocomposite films is the angle measured from the tangent made to the drop curvature at the base.

Bioactivity experiments in simulated body fluid (SBF)

The bioactivity of PSf/n-SHA nanocomposites was evaluated by immersing the samples in SBF to monitor the formation of apatite on the surface of samples. The SBF was prepared and buffered at pH 7.4 at 37 °C with tris-(hydroxymethyl)-aminomethane [(CH$_2$OH)$_3$CNH$_2$] and hydrochloric acid (HCl). The concentration of different ionic species in SBF closely resembles with that of human blood plasma and all PSf nanocomposites were immersed in SBF for 7 days at 37 °C. After soaking, samples were removed from SBF, gently washed with deionized water, and dried at room temperature. The surface morphology of the composites after soaking in SBF solutions was characterized using AFM to confirm the formation of apatite layer.

Protein adsorption

The adsorption experiments were made with bovine serum albumin (BSA), human plasma fibrinogen (FNG) and platelet poor plasma (PPP). The concentrations of BSA and FNG were 4.0 g/dl and 0.3 g/dl in phosphate buffered saline (PBS, pH=7.4), respectively. The membranes with an area of 1x1 cm$^2$ (each piece) were incubated in distilled water for 24 h, washed 3 times with PBS solution, and then immersed in the protein solution for 2 h. After protein adsorption, the nanocomposite films were carefully rinsed 3 times with PBS solution and then rinsed with distilled water.

RESULTS AND DISCUSSION

ATR-FTIR analysis

![Fig.1.FTR patterns of n-HA, and n-SHA](image)

Fig.1 shows the FTIR patterns of n-HA, and n-SHA. The spectrum of n-HA shows a band at 3570 cm$^{-1}$ due to free -OH group stretching vibration. The absorption peaks at 1037 and 962 cm$^{-1}$ are related to stretching vibrations of phosphate group, and peaks at 567 and 603 cm$^{-1}$ are related to deformation vibrations of phosphate group. The peaks at 1411 and 1448 cm$^{-1}$ indicate carbonates. In n-SHA, the new band at 1549 cm$^{-1}$ is ascribed to antisymmetric stretching vibration of COOCa from the interaction between COOH and Ca$^{2+}$, and the one at 879 cm$^{-1}$ assigned to HPO$_4^{2-}$ group coming from reaction of PO$_4^{3-}$ with H$^+$ from COOH group. The new absorption bands at 2924, 2854 are due to CH$_3$ and CH$_2$ groups coming from stearic acid molecule [8].

X-ray diffraction analysis
The XRD patterns of n-HA and n-SHA are shown in Fig. 2. The HA nanoparticles exhibit several sharp peaks at 2θ regions of 26°, 32–34°, 40°, and 46–54° which are consistent with the crystalline nature of HA nanoparticles. The crystalline phases of HA nanofillers before and after modification with SA is found to be same. This confirms that the modification with stearic acid does not cause any crystalline change and secondary phases formed.

**Transmission electron microscopy**

The TEM images of the n-HA and n-SHA dispersed in alcoholic medium are shown in Fig. 3. It shows that the nanoparticles have rod like structure with about 50-70 nm in length and 20-30 nm in width. The dispersion of HA nanoparticles after modification is obviously better than that before modification. Hence, improved compatibility is expected between n-SHA filler and PSf matrix.

**AFM images of the nanocomposites**

AFM is used to understand the nanofiller dispersion in the polymer matrix which distinguishes filler aggregates and single filler particles in the polymer matrix with the surface morphology. In Fig. 4(a-d), the AFM images are different of the composites with filler concentration 0, 1, 3 and 5% are given. Fig. 4a shows the image of the neat matrix. As seen in the figure the neat PSf surface is smooth and homogenous. In the images of the filled composites, we can see the nanofillers in the matrix as globules and its size is increasing from the lower loading to higher loading. The nanocomposites with 1% and 3% loading clearly show more individualized and uniform distribution of filler with the size of 40–60 nm without any remarkable agglomeration. When the filler content reached 5%, many aggregates on a micrometer scale were observed on the surface of the nanocomposites.

<table>
<thead>
<tr>
<th>Nanocomposite</th>
<th>RMS (R_q) (nm)</th>
<th>R_s (nm)</th>
<th>R_q - R_s (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS0</td>
<td>3.02</td>
<td>2.22</td>
<td>0.80</td>
</tr>
<tr>
<td>PS1</td>
<td>5.39</td>
<td>3.85</td>
<td>1.54</td>
</tr>
<tr>
<td>PS3</td>
<td>6.62</td>
<td>4.97</td>
<td>1.65</td>
</tr>
<tr>
<td>PS5</td>
<td>10.39</td>
<td>7.80</td>
<td>2.59</td>
</tr>
</tbody>
</table>

The surface roughness of bone implant has a significant influence on the adhesion and proliferation of osteoblasts [9]. The changes in the surface topography of nanocomposite could be determined quantitatively in terms of surface parameters, such as the average roughness, R_s and the root mean square (RMS) roughness, R_q. The R_q and R_s values of composites are given in Table 1. In case of the neat PSf, R_q value was found to be 3.02 nm and R_s was 2.22 nm. Generally, R_q and R_s values will be almost equal, Table 1 Roughness parameters of PSf nanocomposites

If there is no large deviation from the mean surface level. The difference between R_q and R_s value of 0.80 nm suggests that there is not much undulation for neat matrix. It can be seen that all the nanocomposites show higher (R_q - R_s) values compared to that of neat polymer, which confirms the presence of filler particles on the surface. It was found that roughness parameters increase with filler loading and show maximum
value at 5%. Since natural bone has a nano rough surface consisting of nanosized hydroxyapatite and collagen molecules, producing nano rough surfaces on these implants is more favorable for early osteoblast adhesion and growth on nanocomposite surface, which improves orthopedic implant efficacy [10].

Mechanical properties

The mechanical properties of nanoceramic/polymer composites depend strongly on the size and shape of ceramic nanoparticles, dispersion of nanoparticles, particle loading, interfacial adhesion between the ceramic and polymer phase, and inherent properties of the polymer matrix. So the change in these properties with varying filler content can be taken as an alternative method to evaluate the dispersion of nanofiller in the polymer matrix [11].

Table 2. Mechanical properties of the samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tensile strength (MPa)</th>
<th>Young’s modulus (MPa)</th>
<th>Elongation at break %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PES0</td>
<td>4.72</td>
<td>147</td>
<td>33</td>
</tr>
<tr>
<td>PES1</td>
<td>5.33</td>
<td>170</td>
<td>45</td>
</tr>
<tr>
<td>PES3</td>
<td>5.97</td>
<td>190</td>
<td>57</td>
</tr>
<tr>
<td>PES5</td>
<td>5.10</td>
<td>211</td>
<td>34</td>
</tr>
</tbody>
</table>

The mechanical properties of the PSf nanocomposites with varying filler content were characterized by tensile tests, and thus tensile strength, Young’s modulus and the elongation at break were calculated. From Table 2, it can be seen that mechanical properties like tensile strength and elongation at break of nanocomposites show a tendency to increase and then decrease with increasing n-SHA content and have maximum value at 3% filler content. The highest value of tensile strength and elongation at break obtained for the nanocomposite with 3% of filler loading is due to the good dispersion and the improved interfacial adhesion between nanofillers and PSf matrix, which results in the effective transfer of applied stress to the particles from the matrix [12]. The more uniform the nanoparticle dispersion in the polymer and the stronger interaction between the nanoparticles and polymer matrix, the more improved the mechanical properties. The potential explanation for the decrease in the tensile strength after optimum filler content (3%) is mainly due to the agglomeration of n-SHA particles, which was evidenced from the morphological analyses. In 5% nanocomposite, discontinuity in the form of debonding exists because of non-adherence of nanofillers to the polymer and the stress transfer at the polymer/n-SHA particles interface becomes ineffective. Young’s modulus, expressing the stiffness of the material, show noticeable improvement, with addition of nanofiller to PSf. It is observed that, for the addition of 5% of n-SHA, around 55% increase in modulus occurred. It is well known that the modulus increases for a polymer when we incorporate any mineral filler into it. This is reasonable because the rigid inorganic fillers have higher stiffness values than the organic polymer [13]. The ability of nanocomposites to tailor the mechanical strength by varying the filler content makes it a better candidate material for more effective orthopedic applications from a mechanical perspective.

Contact angle studies

Contact angle measurements carried out with water are often used as an empirical indicator of wettability of PSf/n-SHA nanocomposites. The contact angle of pure PSf was 85°. With the addition of 1, 3 and 5 wt% nanofiller, the contact angles increased to 87°, 92° and 95° respectively, which indicates that the presence of n-SHA in the PSf matrix improves the hydrophobicity of the nanocomposite surfaces. The increase in hydrophobicity is mainly attributed to the difference in both the chemical properties of polymer surface and its surface morphology. In order to have a homogenous dispersion in hydrophobic polymeric host, n-HA surface was modified with SA, which lowers the hydrophilicity of filler surface. It has been recognized that in a multi-component polymer system, the surface composition may differ from that in the bulk since the components of lower surface energy always tend to enrich the surface in order to minimize the free energy of the system. So, in the PSf nanocomposite system, lower surface energy n-SHA components migrate to the surface due to the difference in
the surface energy of the components. Thus, the nanocomposite surfaces become more hydrophobic relative to the neat polymer due to the formation of film surface with lower equilibrium surface energy. The correlation between AFM surface analysis and water contact angle measurements indicated that the increase in surface roughness increases the hydrophobicity [14].

Simulated body fluid test

The essential requirement for an artificial orthopedic material to bond to living bone is the formation of bone-like apatite on its surface when implanted in the living body [15]. Therefore, the apatite forming ability of the implants was studied by soaking in simulated body fluid (SBF) with ion concentrations nearly equal to human blood plasma. AFM images of nanocomposites after soaking in SBF for 7 days are presented in Fig. 5(a-d). This allows the comparison of nanocomposite surfaces with varying filler concentration before and after soaking in SBF. The white deposits on the surfaces confirm the formation of apatite layer. In the case of unfilled samples, some scattered and discrete spherical deposits were seen on the surface. But in nanocomposites, the spherical deposition increased with the increasing filler content. When the filler content reached 5%, these deposits cover the entire composite surface and large aggregated deposits were found at different areas of the composite surface, which is not in the case of composites with low filler content. This suggests that the large globules might have been formed by a secondary nucleation mechanism onto nanosized calcium phosphate entities initially formed. The nucleation of apatite should be easier on a surface of the same nature (apatite) compared to the nucleation on a chemically different surface like polymer nanocomposite. Even the surface roughness has no pronounce effect on the initial heterogeneous nucleation of apatite, rough topography of 5% nanocomposite is beneficial for the further growth and mechanical attachment of apatite coating on the implant surface [16].

Protein adsorption studies

Topographical AFM images of PSf nanocomposites after protein adsorption are depicted in Fig.6. The presence of BSA molecules on implant surface is confirmed from the spherical features seen on the surface after BSA addition. The thickness of the layer denotes the amount of adsorbed BSA and the comparison between the images revealed that extend of adsorption increases with filler content. The positive correlation between extent of protein adsorption and the hydrophobicity of nanocomposite surface suggest that the hydrophobic interaction is a major driving force for adsorption [17]. Proteins tends to adsorb more extensively and less reversibly at hydrophobic surfaces than at hydrophilic surfaces. With increasing degree of hydrophobicity of the surface, the ease of exchange of adsorbed protein molecules with the bulk aqueous phase is generally reduced. This difference can be attributed to a greater degree of unfolding at hydrophobic surfaces following instantaneous protein adsorption, which leads to the development of strong interfacial hydrophobic interactions and associated displacement of vicinal water molecules from the unfavorable environment of the surface. This explains the rather general experimental finding that in most cases the affinity of proteins to surfaces increases on hydrophobic substrates and decreases on hydrophilic substrates. Further, increased adsorption with filler content is also related to its high surface roughness and according to Elimelech et.al increase in roughness favors the protein to transport preferentially to valleys on the nanocomposite surface. However, the uniform distribution in the nanocomposites minimizes the bilateral disulphide bond formation among the protein.

Fig.5.AFM images of nanocomposites after soaking in SBF
molecules and which leads to the more aligned cake layer protein on the surface. The results from these experiments serve as a guide to tune protein adsorption behavior of polymer nanocomposite surface as a function of filler composition and to understand the role of nanomorphology in cell polymer nanocomposite interactions.

CONCLUSION

The combination of PSf with variable amounts of stearic acid modified nano hydroxyapatite for investigating the mechanical, thermal, hydrophobic and biocompatible properties were prepared using solution casting. AFM measurements showed the presence of filler particles on the surface of nanocomposites which caused the increase in $(R_q - R_a)$ values compared to the virgin polymer. The mechanical properties of the nanocomposites like tensile strength and elongation at break showed improvements at lower filler content and decreased with higher filler loading while Young’s modulus showed increase with respect to the loading. The hydrophobic properties of polymer nanocomposites were enhanced by the incorporation of $n$-SHA, as evidenced by increased contact angle. The nanocomposites induced a dense and continuous layer of apatite, after soaking them in simulated body fluid (SBF) for 1 week. The increased surface area and nanoscale surface features of nanomaterials provide more available sites for protein adsorption on the nanocomposite surface with increasing filler content. All these results indicate that the PSf nanocomposites fulfill the basic requirements of a bone implant and have the potential to be applied in bone tissue replacement and regeneration applications.

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