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Physical and cytocompatibility properties of bioactive glass–polyvinyl alcohol–sodium alginate biocomposite foams prepared via sol–gel processing for trabecular bone regeneration

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Abstract In the present work, biocomposite foams of bioactive glass along with polyvinyl alcohol and sodium alginate are designed and developed as a potential biomaterial for bone regeneration. These biocomposite foams have a low density of 0.92 g/cm^3, providing desired property for bone tissue engineering applications. Biocomposite foams were prepared via surfactant foaming. Scanning electron microscopic characterization revealed pore size of 200–500 μm of the biocomposite foams. When these materials were incubated in simulated body fluid, hydroxyapatite layer formation was observed on the material surface. To confirm the cell viability and proliferation on these materials, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed with NIH 3T3 fibroblast cells and the results revealed good biocompatibility with the biocomposite foams. Cell adhesion studies further confirmed the biocompatibility of the scaffolds via cell attachment and ECM production. The optimally synthesized biocomposite foams had a good combination of physical properties with compressive strength of 1.64 MPa and elastic modulus of 18 MPa. In view of the favorable combination of physical and biological properties, the newly developed materials are considered to be suitable for regeneration of trabecular bone.

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1 Introduction

Bone is the second most transplanted tissue after skin. The current status of strategies for bone transplantation is; 45% autologous bone, 45% allogenic bone and 10% tissue engineered bone [1]. Although autografts are considered as gold standard for bone transplantation, yet there are many problems associated with autografting such as bulk limitations, graft donor site morbidity and lengthening of the procedure during the process of harvesting. Similarly, allografts are also associated with problems like cost, availability, antigenicity, infectivity, reproducibility and structural stability. Due to the lack of availability and the number of problems associated with autografts and allografts, recently, the development of tissue engineered bone is considered to be suitable for tissue repair or regeneration.

Bone is a dynamic and highly vascularized tissue that continues to remodel throughout the lifetime of an individual [2]. Bone tissue itself can be categorized into two kinds of arrangements, i.e., compact pattern (cortical bone) or a trabecular pattern (trabecular bone). In maximum extensive efforts to develop materials for bone replacement, number of materials have been investigated in last few decades, e.g., some of these materials are ceramic–metal [3], only bioceramics, and glass–ceramic [4]. Although these materials have been used for bone replacement/regeneration, but they all have certain limitations and drawbacks which are as follows: (a) metal implants generally corrode with time and lack bioactivity, (b) inert ceramics also lack bioactivity, (c) bioactive ceramics and bioglasses do not match the mechanical properties of bone.

On considering these limitations associated with the representative materials, researchers have narrowed their

choices to biocomposites as the preferred material for bone tissue engineering. Most natural biological materials are polymeric composites. To this end bone is a typical example, which is a composite of collagen (protein) and hydroxyapatite (ceramic). Biocomposite materials contain at least two different categories of the materials and such materials have a potential to produce a light weight and high strength device with anisotropic properties similar to bone. Natural and synthetic polymers/co-polymers as well as their composites are widely used as scaffolds for tissue engineering [5, 6], but due to the compliant nature and poor mechanical properties, these polymers have not been much explored for bone regeneration. Till date, various composites of polymer and inorganic components have been developed for bone tissue engineering, e.g., HDPE (high density polyethylene)–Al_2O_3–HAp composites [7], co-poly(methylmethacrylate (MMA)-vinyltriethoxysilane (VTS)) with tetraethoxysilane (TEOS) [8], chitosan–alginate [9], gelatin–siloxane [10], TEOS–PVA (polyvinyl alcohol) [11], etc. In the case of bone tissue engineering, materials should preferably be both *osteoinductive* (capable of promoting the differentiation of progenitor cells down an osteoblastic lineage), *osteoconductive* (support bone growth and encourage the ingrowth of surrounding bone) and capable of *osseointegration* (integrate into surrounding bone). The inorganic–organic biocomposites, which are targeted for mimicking the natural bone, should combine the toughness of a polymer phase and the strength of an inorganic phase to form materials with improved strength and degradation profiles.

Among various processing approaches, sol–gel processing is an interesting route that can combine inorganic/organic components at the nanoscale (e.g., creating a network from synthetic or biological polymers and inorganic silica chains) [12, 13]. Recreating the same degree of nanoscale order in the organization of the mineral and organic components as found in vivo, however, is a challenging task. Mechanical properties of the available composites still fall short of that of bone nor do they attempt to match its anisotropy.

In this work, the development of a biocomposite material via sol–gel method of processing, with the properties falling in the range of materials for trabecular bone is reported. The inorganic part (ceramic component) used for synthesis of such biocomposite is tetraethylorthosilicate along with calcium oxide, whereas the organic part (polymeric component) comprises of sodium alginate and polyvinyl alcohol. Tetraethylorthosilicate (TEOS) is selected as the inorganic component, because silicate glasses have shown immense efficiency as bone tissue engineering materials, since they have osteoconductive, osteoinductive and osteointegrative potential in the presence of calcium component, despite having a brittle nature [14, 15]. This TEOS along with CaO acts as the bioactive glass component in the composite. These bioactive glasses are widely used as scaffolds for bone tissue engineering due to their various favourable properties related to bone tissue engineering [16–18]. Polyvinyl alcohol (PVA) is selected as the synthetic polymeric material, because the polar nature of polyvinyl alcohol facilitates the formation of hydrogen bonds and eventual condensation with silanol groups (from developing polysilicate network) formed by hydrolysis of the silicon alkoxides. Moreover, PVA has been proposed for controlled release systems and is employed in a variety of biomedical applications, generally being considered to be biocompatible. Although not a biodegradable polymer itself, when associated with a biodegradable sol–gel derived bioactive glass, PVA molecules are expected to be eliminated by the body. Sodium alginate, used as the third component, is a biodegradable and biocompatible polymer and has been used for clinical reconstruction of bone [9]. While selecting the material, we have also considered the individual melting point and the mechanical properties of the material, so that the final composite provides high mechanical strength and can withstand high processing temperatures taking into account of the polymeric components. Therefore, in the present work, we report our initial effort to develop bioactive glass–polyvinyl alcohol–sodium alginate biocomposite foams for trabecular bone regeneration.

2 Materials and methods

2.1 Materials

The materials used for the synthesis of biocomposite are sodium alginate (Loba Chemie, India), tetraethylorthosilicate (Merck, Germany), polyvinyl alcohol (Mol. Wt. 72,000; Merck, Germany), nitric acid (Qualigen Fine Chemicals, India), hydrofluoric acid (Rankem, India), calcium oxide (Merck, Germany), sodium lauryl sulphate (SRL, India), Dulbecco's modified Eagle's medium (Sigma–Aldrich, USA), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma–Aldrich, USA), sodium bicarbonate (Ranbaxy, India), fetal bovine serum (Invitrogen, USA), penicillin–streptomycin antibiotic (Sigma–Aldrich, USA) and trypsin–EDTA (Sigma–Aldrich, USA). All the starting materials are of high purity grade.

2.2 Sol–gel synthesis and consolidation

Sol–gel method of synthesis was applied for the preparation of sol mixture [19]. TEOS (70.5% of total weight) was mixed in deionized water in 1:12 molar ratio and 2N nitric

acid (1% of total volume) was added. After stirring for around 1 h, a clear sol solution was formed. Subsequently, CaO (3.5% of total weight) was added to this clear solution and on stirring, instant gelation occurred. After that, the gel was dried and a white powder was obtained upon heating. This dried TEOS–CaO mixture was again resuspended in water, maintaining the initial volume of sol mixture. Finally, a milky solution was obtained. Alginate (7.05% of total weight) solution was prepared separately in 2 ml deionized water. Similarly, PVA (18.9% of the total weight) solution was also prepared separately in 6 ml deionized water by boiling the solution. To the initially prepared TEOS–CaO milky solution, PVA and alginate solutions were added under stirring conditions. After some stirring, HF (hydrofluoric acid) (5 vol%) was added as a gelling agent and stirring was further continued. Finally, a thick white solution of biocomposite was formed. The gelled biocomposite material was aged at 50°C for around 2 days until completely dried and a hard mass of biocomposite material was formed. Further, this material was powdered in mortar and pestle to obtain finest particle size. Subsequently, this powder was molded in pressing machine using a suitable die to obtain cylindrical pellets. Sintering is performed to increase the density and strength of the components. The pellets obtained were sintered at 100°C for 2–3 h. Sintered pellets were much harder as compared to the green body (non-sintered pellets). The foams of the biocomposite material were synthesized using 5% (v/v) sodium lauryl sulphate (SLS) as the surfactant and 3% (v/v) hydrofluoric acid (5 vol%) as the gelling agent in the initially prepared biocomposite solution. This biocomposite solution was foamed for around 30 min by vigorous agitation and then kept in incubator at 60°C for 18 h. These foams were further sintered at 100°C for 2–3 h. The foam samples as obtained were ethanol dried and then kept overnight for vacuum drying. The dried samples were gold coated and then analysed via scanning electron microscopy for their morphology.

2.3 Characterization

The density of the biocomposite pellets and foams was calculated using Archimedes principle. The mechanical properties were calculated from stress vs. strain curve using the mechanical testing machine INSTRON 1195. The samples used for this experiment were of cylindrical shape and were tested with crosshead speed of 0.1 mm/min. The mechanical properties of the biocomposite foams were also calculated by similar method with a crosshead speed of 0.5 mm/min using rectangular shaped samples. Scanning electron microscopy was used to observe the microstructure of the biocomposite pellet and foam. Samples were sputter coated with gold and viewed in FEI Quanta 200 SEM using an accelerating voltage of 20 kV and a spot size of 3 μm. The FTIR spectral studies were conducted using FTIR spectrophotometer in the range of 4000–500 cm^{-1}. The samples were incubated in simulated body fluid (SBF) for 21 days and their EDS (Energy Dispersive X-ray) analysis was done to find if there was formation of HAp-like layer based on the presence of the constituents, i.e., calcium, oxygen and phosphorus. NIH 3T3 fibroblast cells were used for cell culture experiments at a seeding density of 1×10^4 and 1.5×10^5 cells/ml for biocomposite pellet and foam, respectively. The NIH 3T3 fibroblast cells were cultured in scaffolds and then the media was replaced with serum free culture medium containing thiazolyl blue (MTT) (0.5 mg/ml). MTT assay was performed in order to assess the biocompatibility in terms of cytotoxicity of the biocomposite material. Samples were incubated for 4 h in CO_2 incubator at 37°C, further, serum free culture medium containing thiazolyl blue (MTT) was aspirated and 1 ml DMSO was added to each sample and scaffold samples were disintegrated and kept for 10 min. The absorbance of each sample was read at a wavelength of 490 nm by UV–vis Spectrophotometer.

3 Results and discussion

3.1 Density and microstructure

After sintering, the biocomposite pellet samples used for density measurement were of diameter 5 mm and height of 7 mm. The average density of the pellet samples obtained via Archimedes principle was 1.57 g/cm^3. It can be noted that the trabecular bone tissues from a large number of cadavers and live organisms are reported to cover a wide range in apparent density (0.09–0.75 g/cm^3), thus providing a broad picture of human trabecular bone elastic behavior [20]. In the present case, the density of the pellet material is much higher as compared to that of the trabecular bone and upon foaming they may well match the density of trabecular bone. To further confirm this, the density of the foam materials was calculated and the average density of the foam scaffolds was found to be 0.92 g/cm^3. Once present in the body, the material is expected to start degrading and will lead to further decrease in the density of the material due to the increase in the size and number of pores. Therefore, the density is low enough to mimic the trabecular bone whose density lies in the range of 0.09–0.75 g/cm^3.

SEM images of polished biocomposite pellets reveal a rather dense microstructure (Fig. 1a). The presence of inorganic phases (Fig. 1b) appear in brighter contrast. SEM image of foam scaffolds was also observed and around 1×1 cm samples were gold coated and viewed for their

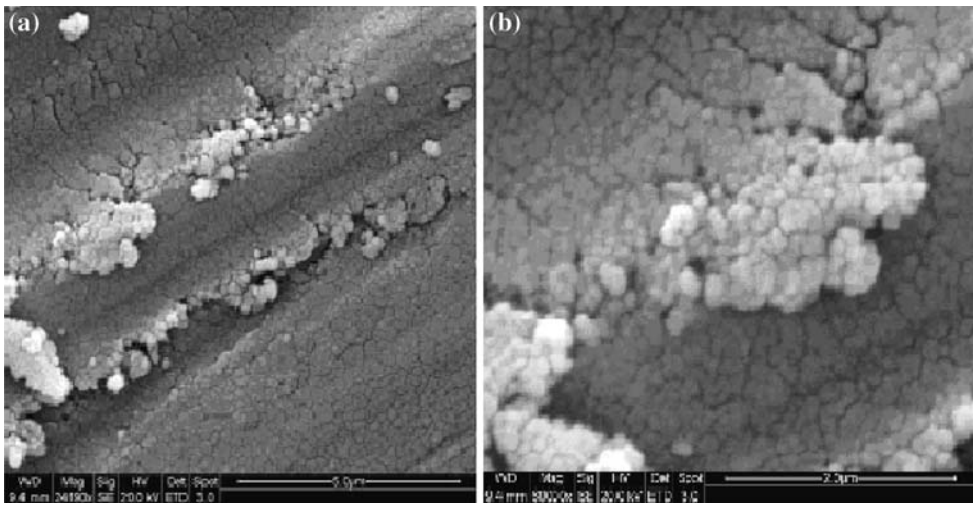

Fig. 1 SEM images of the biocomposite material revealing the dense morphology (**a**) and the presence of inorganic phases in the brighter contrast (**b**)

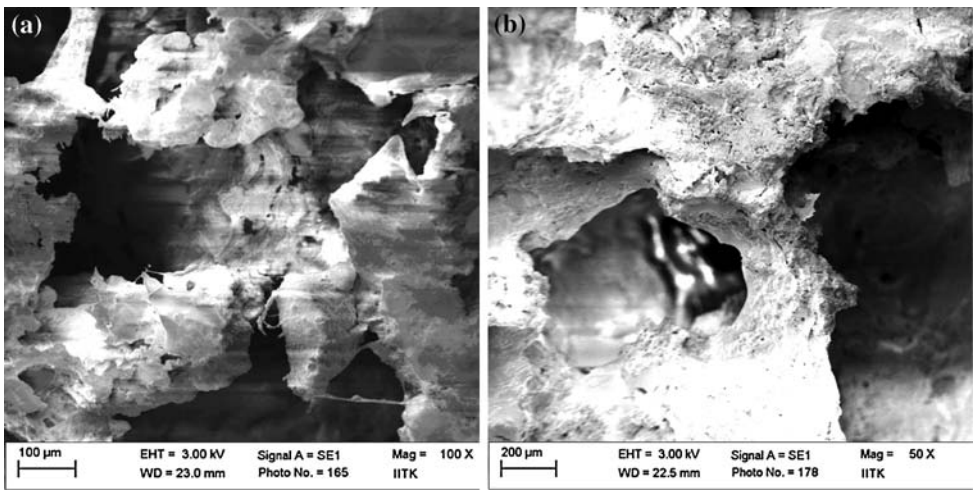

Fig. 2 SEM analysis of the biocomposite foam synthesized by foaming with surfactant, showing a pore size of around 200–500 µm. **a** The overall pore morphology and **b** single interconnected pore

morphology. SEM images clearly indicate a rough morphology of the foamed samples with a pore size of 200–500 µm, as shown in Fig. 2a, also the pore size can be clearly observed along with the interconnectivity in the case of Fig. 2b. Such large pores should, in principle, encourage bone cell adhesion and proliferation. It can be mentioned here that the ideal pore size for bone tissue engineering is in the range of 200–800 µm. The FTIR studies (Fig. 3) reveal a characteristic broad peak of –OH stretching at 3440 cm^{-1} and such peak is obtained at higher concentration of –OH group, as found in the case of PVA. The peak of –CH– stretching of alkanes was recorded at 2942 cm^{-1}. The characteristic peaks at 1632 and 1426 cm^{-1} correspond to $–COO^-$ group of sodium alginate. The peak at $\sim$1100 cm^{-1} is related to Si–O stretching vibration of tetraethoxysilicate gel matrix, while the peaks obtained at $\sim$800 and $\sim$500 cm^{-1} correspond to the Si–O–Si bending vibration of tetraethoxysilicate gel matrix.

3.2 Mechanical properties

The evaluation of compressive strength was necessary in order to compare the mechanical strength of the biocomposite pellet and foam with respect to that of the human trabecular bone. Figure 4a, b plot the stress vs. strain behavior of the biocomposite pellet and foam respectively, during compression testing. In case of pellets, upto stress level of 20 MPa, a non-linear stress–strain response was recorded and thereafter, a linear response, i.e., elastic region was extended till 80 MPa. Subsequently, the load bearing capability decreases, causing failure of the samples. The average compressive strength calculated from a number of tests on the biocomposite pellet samples was found to be 72 MPa. From the slope of elastic region, the average modulus of elasticity of the material was estimated to be 0.76 GPa, while the modulus of elasticity of trabecular bone lies in the range of 0.02–0.5 GPa. The fracture strain of the

Fig. 3 FTIR plot of the biocomposite material showing peaks corresponding to constituents of the biocomposite after sintering at 100°C

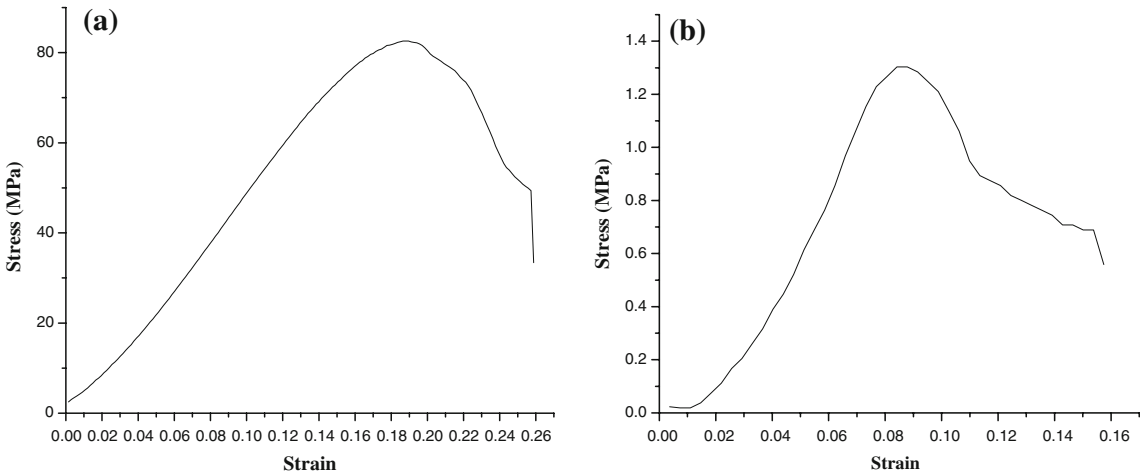

Fig. 4 Representative stress vs. strain plot, **a** with the biocomposite pellet under compressive loading at a crosshead velocity of 0.1 mm/min and **b** with the biocomposite foam under compressive loading at a crosshead velocity of 0.5 mm/min

biocomposite pellet was calculated to be $20 \pm 2\%$. A study reveals that fracture occurs in case of trabecular bone at small strain of around 0.20–0.45% [21]. Fracture strain determines the ductility of the biocomposite samples, i.e., maximum strain tolerated by the sample before undergoing fracture. The more is the fracture strain, more ductile is the material. Thus, the present biocomposite pellet is more ductile as compared to that of trabecular bone, although the ductility can reduce significantly on preparation of foams of such materials. Similarly, the compressive strength and modulus of elasticity was calculated for foam samples and the average values were 1.64 MPa and 0.018 GPa, respectively. The compressive strength of trabecular bone lies in the range of 2–12 MPa and the modulus of elasticity lies in the range of 0.02–0.5 GPa. Considering these values, it is supposed that the developed biocomposites foams can well suit the mechanical properties of trabecular bone since its mechanical properties closely resembles the lower range of trabecular bone. Table 1 compares the properties of bioactive glass–polyvinyl alcohol–sodium alginate biocomposite pellet and foam with human trabecular bone.

Table 1 Comparison of properties of human trabecular bone relative to the bioactive glass–polyvinyl alcohol–sodium alginate biocomposite

Property	Bioactive glass–polyvinyl alcohol–sodium alginate biocomposite		Human trabecular bone
	Pellet	Foam	
Density	1.57 g/cm^3	0.92 g/cm^3	0.09–0.75 g/cm^3
Compressive strength	72 MPa	1.64 MPa	2–12 MPa
Elastic modulus	0.76 GPa	0.018 GPa	0.02–0.5 GPa

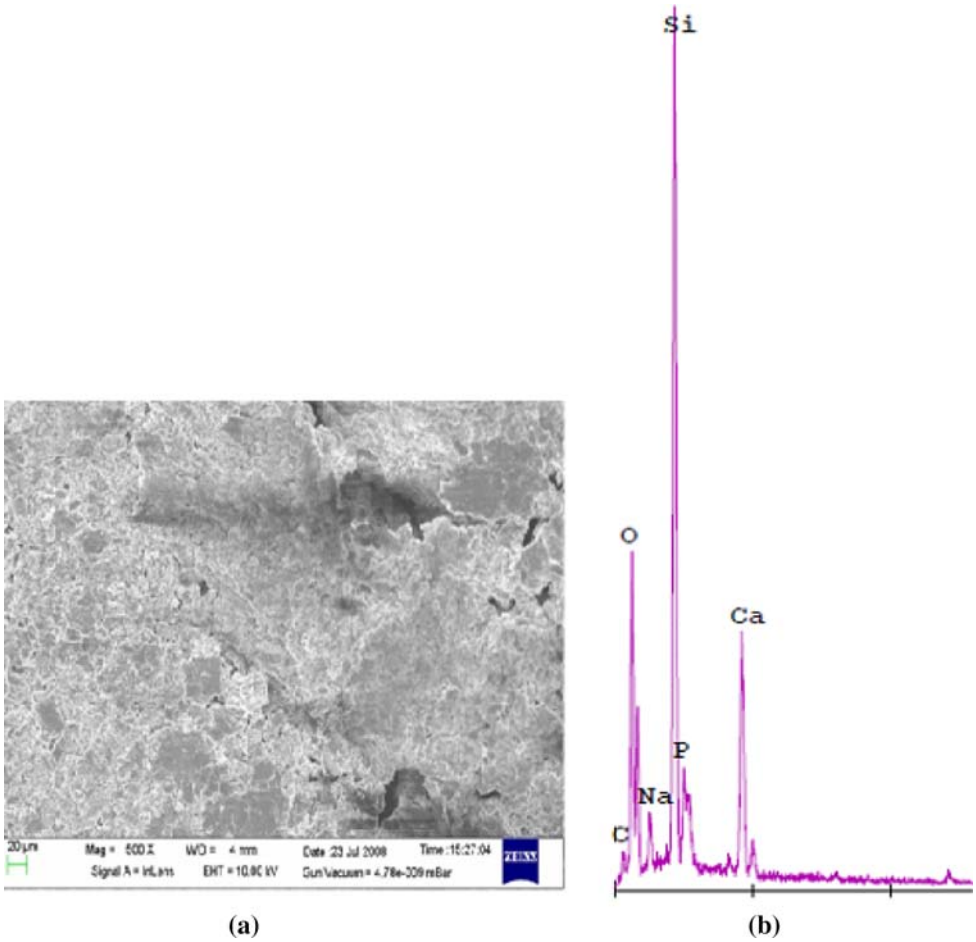

Fig. 5 **a** SEM image of the biocomposite pellet after immersion in SBF for 21 days (length of the bar, 20 μm) and **b** EDS analysis of the same sample

3.3 In vitro properties

3.3.1 Hydroxyapatite (HAp)-like layer formation

The formation of HAp (stoichiometric composition—$Ca_{10}(PO_4)_6(OH)_2$)-like layer on the surface of a biomaterial is beneficial when used for bone tissue engineering applications. This is because of the fact that, in situ formed HAp-like layer mimics the extracellular matrix of bone and therefore helps in forming a strong bond between the scaffold and the natural bone on implantation. In the present case, the material surface was investigated after in vitro dissolution experiments at different time scales. SEM analysis of the surface of the biocomposite pellet confirmed the formation of HAp-like layer on the material surface (Fig. 5a). EDS compositional analysis revealed strong presence of Si, which comes from base glass of the biocomposite material as well as significant presence of calcium, phosphorus and oxygen peaks (Fig. 5b). This also indicates the formation of HAp-like layer on the surface of biocomposite pellet. Looking at the composition of biocomposite as well as considering the SBF composition [22], it should be clear that such layer formation is induced by the chemical dissolution/reaction between biocomposites and SBF.

3.3.2 Cell viability

MTT assay is a quantitative colorimetric assay for mammalian cell viability and cell proliferation. It allows assessing cell growth and proliferation indirectly, since mitochondria oxidizes the MTT solution, giving a typical blue–violet end product. In the case of biocomposite pellets, the samples were incubated for varying time period upto 10 days. The results of MTT assay are plotted in Fig. 6. On assessing the viability and proliferation using the initial cell density of 1×10^4 cells/ml, it was found that the cells were well acclimatized with the matrix and showed an increase in cell number on the scaffold with time, although this increase was much less as compared to the positive control values. The values were supposed to be less as compared to that of control, because the biocomposite pellets are non-porous materials and therefore cells can only attach and proliferate on the surface of the pellets and not inside the material. The above results at least confirm the bioactive potential of the scaffolds as a

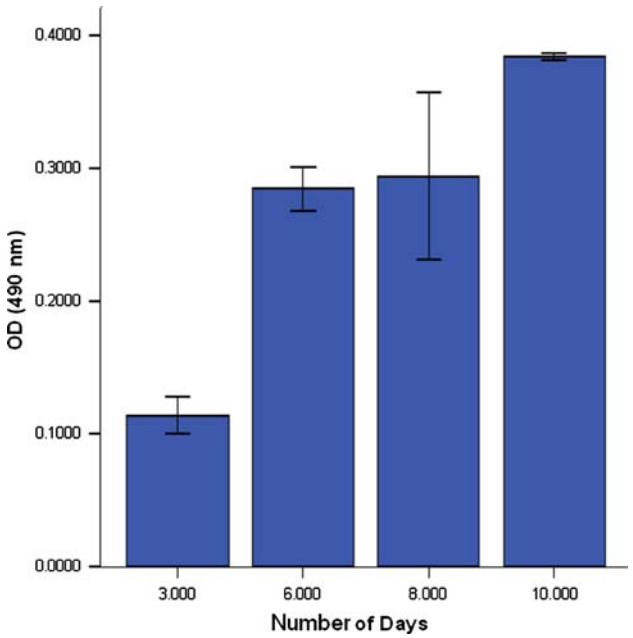

Fig. 6 The OD of fibroblast cells (NIH 3T3) at 490 nm wavelength, on performing MTT assay on the biocomposite pellet, is plotted against the number of days of incubation (error bars indicate ± 1 standard error)

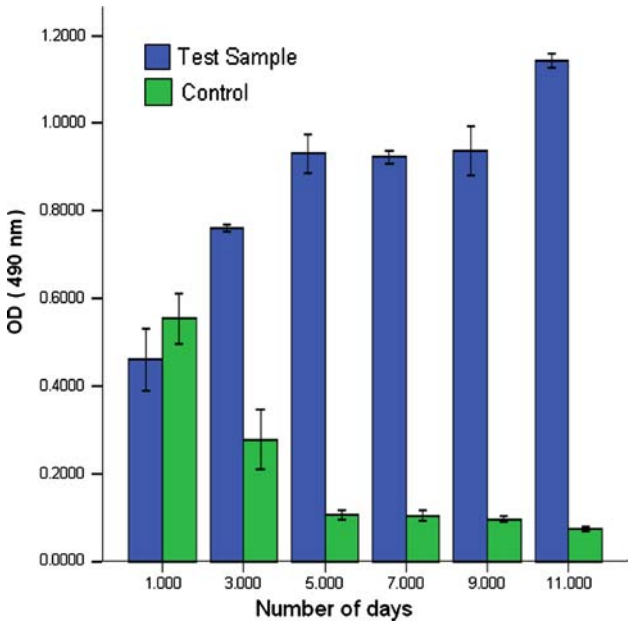

Fig. 7 The OD of fibroblast cells (NIH 3T3) at 490 nm wavelength, on performing MTT assay on the biocomposite foam scaffolds, is plotted against the number of days of incubation (error bars indicate ± 1 standard error)

substrate for cell binding. In case of biocomposite foams, 1 × 1 cm samples were incubated for varying time upto 11 days. The results of MTT are shown in Fig. 7. Cell seeding density was 1.5×10^5 cells/ml. The value of absorbance being much higher in foam scaffolds as compared to that of control, confirms that foams are having enough pores and also the pore size allowed fibroblasts to acclimatize and proliferate inside the foam scaffolds.

3.3.3 Cell adhesion studies

The cell adhesion studies were done by incubating the biocomposite foam scaffolds for various time intervals with the NIH 3T3 fibroblast cells at a seeding density of 1.5×10^5 cells/ml. The adherence of the cells was analyzed on the surface of the biocomposite foams at 7 and 11 days of culture (Fig. 8a and b, respectively). The results show that cells adhered well on the scaffolds, confirmed by the stretched morphology of cells and filopodial extensions protruding from the cells, which are observed only when the cells are compatible with the biomaterial.

4 Conclusions

Bioactive glass is well established as an inorganic component for bone tissue engineering due to its high bioactive potential and good mechanical properties. Although, the novel combination of organic components like sodium alginate and polyvinyl alcohol with that of bioactive glass resulted in some interesting properties, making the proposed material as a potentially viable scaffold composition for bone tissue engineering applications. The density of the foam material is low, i.e., 0.92 g/cm^3, which is an important prerequisite for bone regeneration.

The material proposed here seems to be a good combination of strength and compliance as required for bone tissue engineering. Both the inorganic and organic phase is continuous and well mixed with each other due to the sol–gel processing of the precursors. Also, mechanical properties of the biocomposite are good as revealed by the mechanical testing of the biocomposite pellet (compressive strength, 72 MPa; elastic modulus, 0.76 GPa) and foams (compressive strength, 1.64 MPa; elastic modulus, 0.018 GPa).

The microstructure of the biocomposite pellet shows that the inorganic phase is present in the brighter contrast, while the organic phase forms the continuous matrix. Further, the microstructure of the biocomposite foam suggests that its pore size lies in the range of around 200–500 μm and also the pores were interconnected. This pore size range well matches the required pore size range for bone tissue engineering, i.e., 300–800 μm.

The formation of HAp-like layer on the surface provides bioactive property to the scaffold. The HAp-like layer is formed on the surface of the proposed composite and therefore it is implicated that the bioactive potential of the

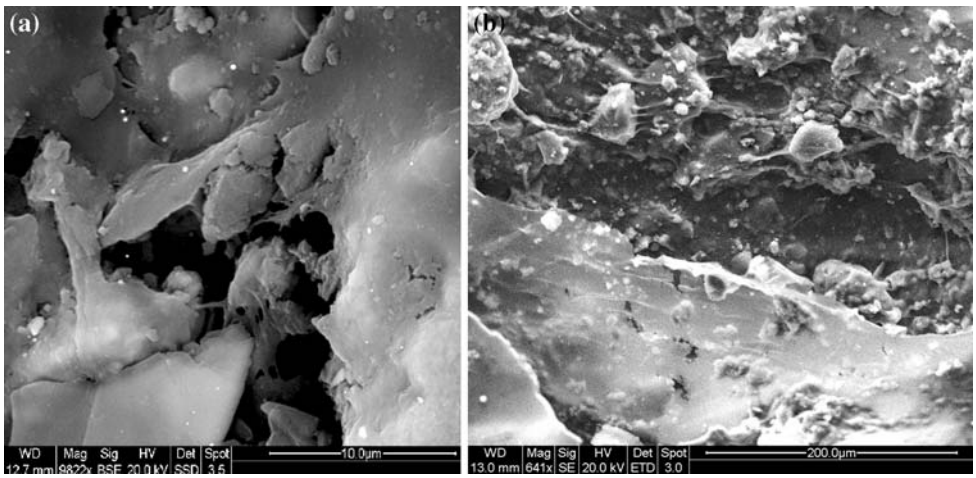

Fig. 8 NIH 3T3 fibroblast cells showing filopodia extensions and flattened morphology at 7 (**a**) and 11 (**b**) days of culture on the foam scaffolds

scaffold will ensure better attachment and proliferation of osteoblasts on the surface of the biocomposite.

The cytocompatibility study using NIH 3T3 fibroblast cells reveal good cell adhesion and spreading. The quantitative assessment of the cell viability using MTT assay indicates that the material is biocompatible under in vitro conditions. Although, further optimizations of the present material may lead to the design of the biomaterial closely mimicking the human trabecular bone tissue.

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