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Biomimetic patterning of polymer hydrogels with hydroxyapatite nanoparticles

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ABSTRACT

We report here an *in situ* process to produce nano-composite polymer hydrogels having surfaces patterned with hydroxyapatite (HA) nanoparticles (100 nm). Poly (vinyl alcohol) (PVA) has been used as a hydrogel forming medium. A three step process, comprising precipitation of HA nanoparticles in presence of PVA molecules and freeze thawing of obtained PVA-HA emulsion, followed by critical point drying, has been devised to produce three dimensional nanocomposite hydrogels. Interaction of Ca^{2+} with oxygen atoms of PVA and the hydrogen bonding characteristic of the polymer have been exploited to have controlled size distribution of HA in a continuous and macroporous network of PVA. A systematic variation in the polymer concentration could be correlated with microstructural features of the hydrogel.

1. Introduction

Biologically produced skeletal structures, such as bones and cartilages, are known for self healing. However, the inherent complexities associated with bone regeneration across the large defects, has motivated materials scientists to find suitable bioactive materials to provide mechanical stability in the defect region and guide tissue regeneration [1]. The ability to develop materials that can interface with tissues structurally, mechanically and biofunctionally is important for the success of tissue engineering [2]. Advances in design and synthesis of nanostructures have paved the ways to develop nanocomposite scaffolds mimicking underlying fibrous structure and specific chemistry of the tissue. Various synthetic and natural materials have been employed for the fabrication of scaffolds, suitable, for cartilage and bone tissue engineering. Employed materials include collagen, hyaluronan, fibrin, poly(lactic acid), poly(glycolic acid), chitosan, demineralised bone matrix and HA [3–9]. Despite the widespread use of materials in tissue engineering, many biomaterials lack the desired functional properties to interface with biological systems [10]. Therefore, there is an increased need to develop new materials to address such issues. Hydrophilic polymers, and specially their crosslinked forms, known as hydrogels, are a class of biomaterials that have demonstrated great potential for biomedical applications [11]. Being physically not very strong, hydrogel scaffolds were initially developed only for soft tissues or non-load bearing bone implants [12]. Recent studies have established that incorporation of growth factors and other biological cues, ranging from inorganic minerals, such as calcium phosphate (Ca-P) and HA, to specific adhesive or degradable sequence in hydrogel, can modulate their performance as a scaffold [13,14]. An attachment of HA nanoparticles to polymer surface has been proposed to promote bone apposition and differentiation of mesenchymal cells to osteoblasts [15]. Attempts have already been made to produce mineralized hydrogel composites for bone tissue engineering. HA and Ca-P have been generally intergraded *ex situ* or *in situ* with supramolecular matrix to form mineralized hydrogels.

It is well known that many of the mineralized tissues are made up of self assembled nanosized building blocks produced by matrix mediated biomineralization [16]. Understanding of underlying mechanism of biomineralization and its adaptation in materials science to develop functional materials and structure is termed as "Biomimetics" [17,18]. *In situ* mineralization of polymers is the simplest model for biomimetic materials [19]. We have already demonstrated that PVA matrix mediated synthesis of HA can lead to different form of PVA-HA nanocomposites ranging from nanopowder to three dimensional sponge like scaffolds [20–23]. Besides the above PVA based scaffolds, urea mediated mineralization of PHEMA, enzymatic mineralization of poly(ethylene glycol), and *ex situ* mineralization of oxidized alginate-gelatine hydrogel are also among the few reported efforts in developing hydrogel based scaffold for tissue engineering [24–26]. Coincidently, most of the above hydrogel mineralization processes lead to the nucleation of HA nanoparticles deep inside the hydrogel, minimizing the scaffold's surface functionality. Keeping the above facts in view, our efforts have been directed towards developing a rapid and effective *in situ* mineralization method that leads to high affinity integration of HA nanoparticles with hydrogel surface. In the present communication, inspired by the process of matrix mediated biomineralization, we report here a PVA mediated process to produce nanocomposite scaffolds having surfaces patterned with HA nanoparticles. An uniform distribution of

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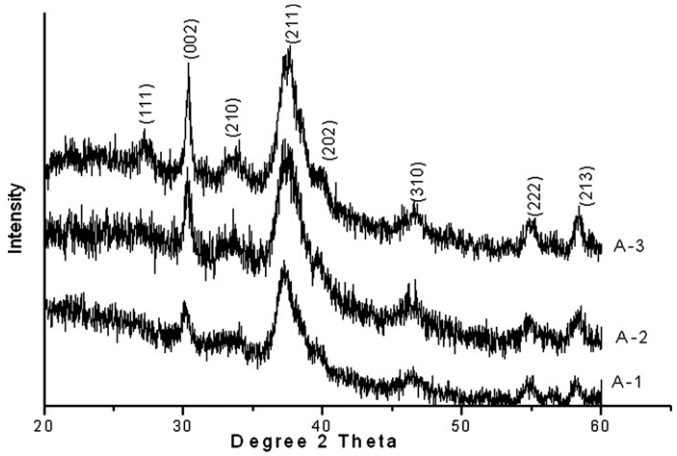

Fig. 1. XRD patterns of PVA-HA hydrogels. A-1 represents 2%PVA-HA, A-2 represents 3% PVA-HA and A-3 represents 4% PVA-HA nanocomposite hydrogels respectively.

nanosized HA particles (100 nm) and their ordered integration on the hydrogel surface, may make this approach useful to produce bioactive scaffolds suitable for tissue engineering applications.

2. Experimental

In the present study, a well known reaction of calcium nitrate with di-ammonium hydrogen phosphate, at an alkaline pH ~10, has been carried out in presence of PVA (molecular weight 125000 and degree of hydrolysis 85-89%, from Qualigens, India) to *in situ* synthesize HA nanoparticles.

In a typical experiment, 75 ml freshly prepared aqueous solution of 0.8 M calcium nitrate tetrahydrate (Hi Media, India) was made alkaline by using 1:2 (17:34) ammonia/distilled water solution and the pH was adjusted to10.5-11. Above alkaline salt slurry was added to the 50 ml of 2% PVA solution and incubated for 24 h at 30 ° C ± 1 ° C for binding calcium ions with PVA. Following that, freshly prepared 175 ml solution of 0.30 M diammonium hydrogen phosphate (Merck, India) was mixed thoroughly with incubated PVA-calcium salt solution. Before mixing with PVA-Ca solution, diammonium hydrogen phosphate solution was made alkaline using 1:1 ammonia/water. Mixing initiated the formation of white precipitate in the solution. System was allowed to age for 2-3 days at room temperature. After aging, entire slurry was incubated at −4 °C for 3 days. Frozen slurry was thawed at room temperature for 1 day and again subjected to freezing for 3 days. This cycle of freezing and thawing was continued for 5 times. Initially, during thawing we observed a solid liquid phase separation on the surface of formed gel. However, after three cycles of thawing, no such separation was observed and after five cycles, we obtained a white coloured, cylindrical shaped, sponge like PVA-HA hydrogel. The process has been repeated again to synthesize PVA-HA nanocomposites with 3 and 4% PVA respectively. Thus obtained three samples have been designated as A-1, A-2 and A-3 respectively.

All the samples were thoroughly washed with distilled water several times to remove possible contaminations and possible Ca-P precipitation unbound to polymer surface. Later, samples were dried by using critical point dryer (Quorum Technology, E 3000). Dry hydrogel samples were later structurally characterized by X-ray diffraction (XRD, Seifert model PTS 3003, target Co-Kα), scanning electron microscopy (SEM, JEOL model JSM 840A), Fourier transform infrared spectroscopy (FTIR, JASCO model FT-IR 410).

3. Results and discussion

Among the notable synthetic polymers, known for hydrogels formation, PVA hydrogels are most acceptable for biomedical applications. Aqueous PVA solutions are known to form chemical gels by γ-ray irradiation, while physical gels are formed by freezing and thawing [27]. Capability of entrapping / chelating cations (like Ca^{2+}, Pb^{2+}, Ti^{4+} etc.) and lack of toxic residues make PVA physical gels, a suitable matrix for developing biomimetic scaffolds [28,29]. XRD studies of nanocomposites synthesized in the present study, confirmed the formation of HA nanoparticles in the PVA gel. All the three samples revealed almost identical diffraction patterns (Fig. 1) comprising broad diffraction peaks pertaining to nanocrystalline nature of inorganic phase present in the polymer matrix. Obtained diffraction peaks could be indexed as (111), (002), (210), (211), (112), (202), (310), (222) and (213) reflections of HA. A poor resolution of (002) and (211) peaks suggest that the phase formed in PVA matrix matches closely with Ca deficient HA (CDHA) [30].

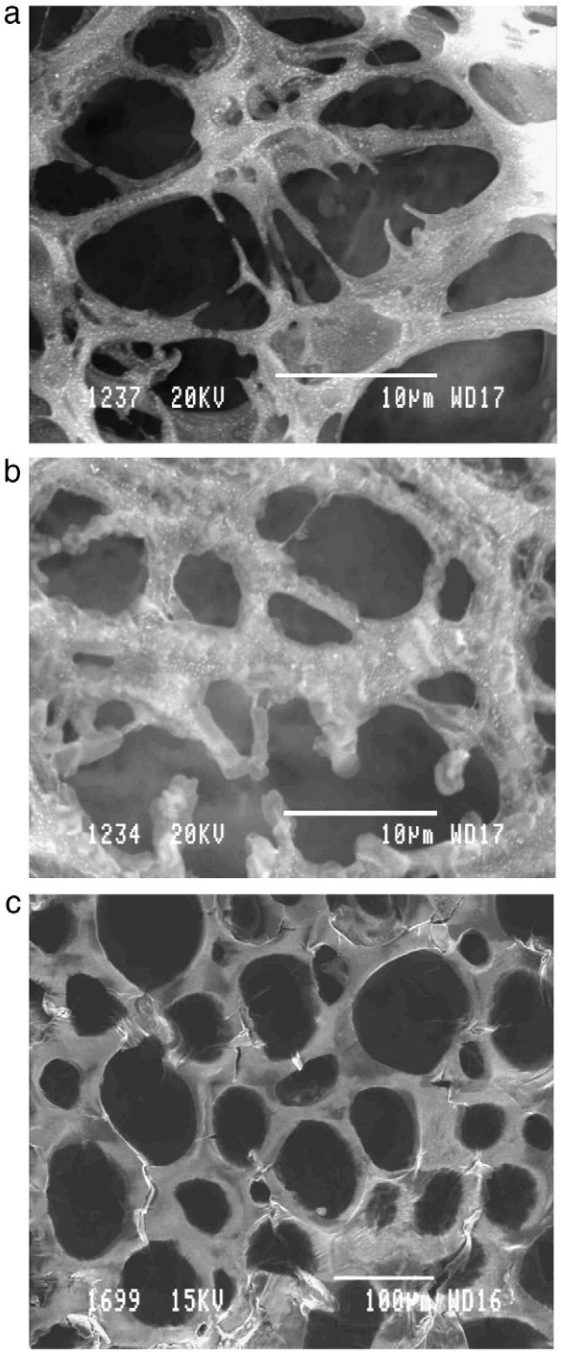

Fig. 2. SEM images of different mineralized hydrogels exhibiting geometry and dimensions of the pores as a function of PVA concentration. (a) represents 2% PVA-HA hydrogel, (b) represents 3% PVA-HA hydrogel and (c) represents 4% PVA-HA hydrogel respectively.

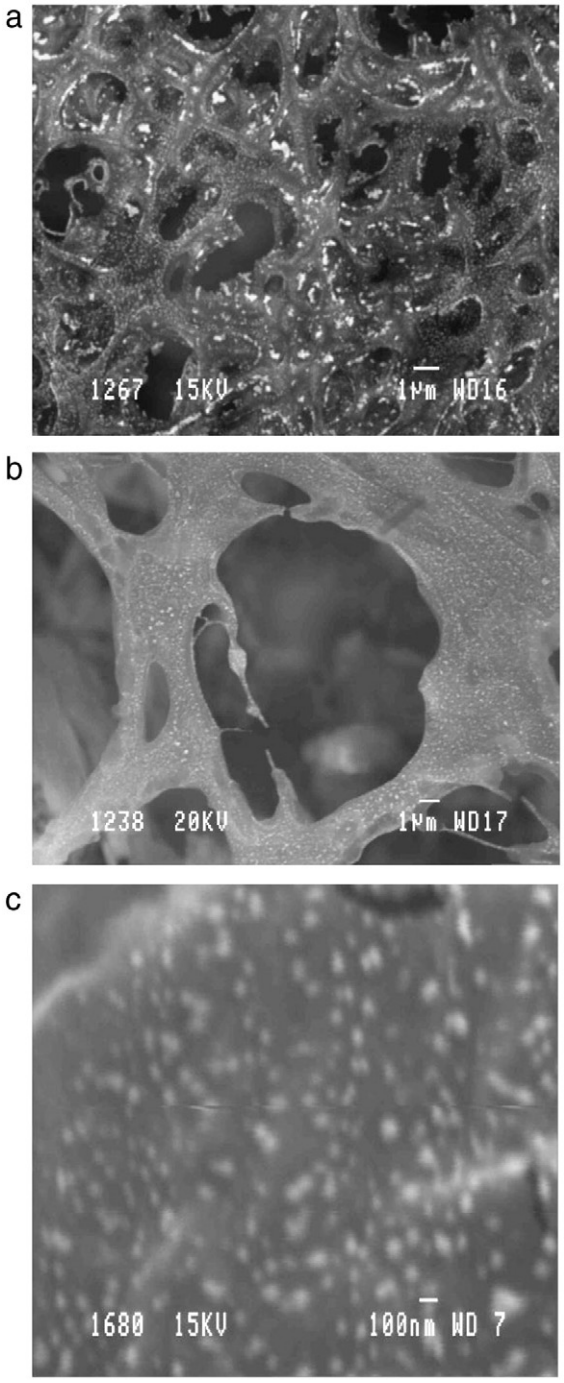

Fig. 3. Higher magnification SEM images of mineralized hydrogels confirming *in situ* nucleation and growth of HA nanoparticles (~100 nm) in three different hydrogels, (a) represents 2%, (b) represents 3% and (c) represents 4% PVA respectively. Images clearly depict bulk mineralization as well as patterning of HA nanoparticles on hydrogel surfaces.

Formation of CDHA is interesting from the point of view that it is very close to physiological HA and may take active part in biological environment. A systematic increase in the polymer concentration from 2% to 4% induced a corresponding increase in the intensity of (002) reflection, indicating the possibility of a preferred growth direction.

It is known that in the process of biomineralization, genetically controlled mineralization kinetics is responsible for creating a range of function specific morphologies of different skeletal structures, comprising identical building blocks. Drawing an analogy, our earlier works clearly demonstrate that by changing the post precipitation treatment of in situ mineralized polymer hydrogel one may produce nanocomposites exhibiting different micro and macrostructures [20–23]. Making a deviation from our earlier employed post precipitation techniques, including oven drying, lypholization and freeze-thawing coupled with vacuum oven drying respectively, the present study involves critical point drying (CPD) of freeze-thaw samples. SEM studies of the samples obtained from CPD revealed uniform mineralization of macroporous hydrogels, achieved through *in situ* nucleation of HA nanoparticles in the bulk as well as on the surface of PVA gels (Fig. 2). Mineralized hydrogels, having PVA concentration 2% and 3% respectively, manifested almost similar microstructures characterized by irregular shaped pores in the size range of 5 μm–15 μm (Fig. 2a and b). However, PVA-HA nanocomposite hydrogel with 4% PVA manifested pores in the size range of 45 μm–100 μm with a relatively regular geometry (Fig. 2c). Higher magnification images of the microstructure of mineralized gels could provide an insight into hydrogel's surface architect and the extent of its mineralization (Fig. 3). It is clearly observed that hydrogel skeleton is composed of randomly bundled PVA micro-fibrils mineralized with HA nanoparticles. Formation of micro-fibrils may be attributed to freeze-thaw driven hydrogen bond induced self assembly and cross linking of PVA tubules present in the solution [31]. An increase of PVA concentration from 2% to 4% has resulted in increased thickness of mineralized bundles from 1.0 μm–15 μm respectively. PVA-HA nanocomposite with 2% and 3% PVA could be characterized with high density of HA nanoparticles (150 nm–120 nm) assembled on the surface of hydrogel matrix (Fig. 3a and b). Similarly, 4% PVA hydrogel's surface manifested patterning with 80 nm–100 nm HA particles (Fig. 3c). A reduction in the dimensions of HA nanoparticles at high PVA concentration may be due to a combined effect of a sluggish diffusion and reduced agglomeration. Higher magnification image of mineralized 2% PVA hydrogel clearly revealed that *in situ* mineralization of PVA hydrogel is a bulk phenomenon and just not limited to surface only.

FTIR study of the three hydrogels also confirmed the formation of nanocomposite structures. All the samples revealed almost similar FTIR spectrum, except that a small increase in the absorbance of PVA specific bands could be seen in 4% PVA-HA hydrogel (Band number 9,10 and 13) and decrease the intensity of the bands associated with HA (Band number 3,4 and 5) (Fig. 4). Absorbance band obtained at 3425 cm^{-1} corresponds to hydrogen bonded -OH stretching band [32]. Bands at 2926.87 and 2850.11 cm^{-1} may be assigned with C-H broad alkyl stretching, while the bands at 2370.32 and 2345.50 cm^{-1} manifest C-H vibration modes. Band at 1666 cm^{-1} is due to C=C stretching mode [33]. Absorbance bands in the range of 1458.10–

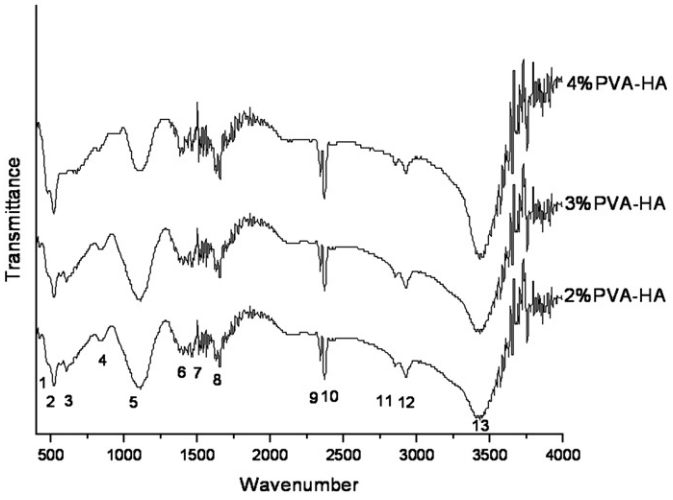

Fig. 4. Comparative FTIR spectrum of three mineralised PVA-HA hydrogels confirming the formation of nanocomposites. Numbers (1–13) correspond to different absorbance bands.

1401.15 cm^{-1} represent both phosphate and CH_2 bonds [34]. Peak around 1113.20 cm^{-1} is due to stretching of P–O and P=O bonds. Similarly absorbance peaks around 844.60, 604.70, 518.34 and 417.18 cm^{-1} were assigned to P-OH bond formation [28,35,36].

Obtained results manifest that the above processes of freeze thawing of mineralised polymer solution to form hybrid hydrogels, has definite advantages over the other approaches. One of the commonly employed processes to form mineralized hydrogels involves dispersion of HA nanoparticles in polymer solution before freeze-thawing. Dispersed HA particles get satirically entrapped between the entangled polymer chains as well as get adsorbed on PVA surface due to non-specific interactions of PVA with HA particles [15]. As there are no interactions involved between the –OH functional groups of PVA and HA nanoparticles, it is difficult to ensure an uniform organic-inorganic integration responsible for functionalized patterned micro-structure. Second interesting process, developed for composite hydrogels for tissue engineering, involves concentration gradient based diffusion of cations / anions from the solution to the gel with or without an enzyme present in the system to act as a catalyst [37]. This process works well as far as the outer surface mineralization of the gel is concerned. However, it has serious limitations in terms of degree of bulk mineralization. Both of the issues of uniform bulk mineralization and systematic integration of HA nanoparticles with PVA chains could be properly addressed in the present process. Uniform distribution of HA nanoparticles in the bulk could be achieved by HA nucleation in liquid phase, while an organic-inorganic integration has been achieved by Ca^{2+} chelation through –OH groups of PVA.

Synthetic and biopolymer mediated synthesis of inorganic nanoparticles and a composite structure has already been established by our group [20–23]. Due to limited solubility of PVA in water, it forms micelle like structures. These micelles undergo self assembly to minimize their free energy by forming tubular structures having few nano-meters diameter and length of few microns. Hydrogen bond assisted assembly of these tubules results in the formation of micro-hydrogels[38]. Presence of Ca^{2+} ions in PVA solution may increases the kinetics of micro-hydrogel formation and such and such a polymer solution (PVA solution containing Ca^{2+} ions), may be modelled as a dispersion of ions loaded microhydrogels. Chelated calcium ions, on further reaction with phosphate ions at an elevated pH, heterogeneously nucleate HA nanoparticles attached to PVA surfaces. HA nanoparticles loaded microhydrogels are under slow molecular motion in the solution. This motion ceases during freezing. With decreasing temperature, size of the ice increases and –OH groups of PVA, not participating in the chelation of Ca^{2+}, undergo hydrogen bonding. Loose crosslinking networks among mineralized microhydrogels are formed in the first freezing and thawing cycle. In other words, it starts translating micro-hydrogels into a macro-hydrogel or bulk hydrogel. Permanent elastic constraints and limited thermal fluctuation around fixed average position in PVA segments controls the overall geometry of the hydrogel. An increase of crosslinking points with further increasing the freezing and thawing cycles strengthens the mineralized PVA hydrogel [39]. It is true that as of now, the present development is more a proof of concept rather than a total solution to bone scaffold biomaterials. It definitely opens up some important issues to be addressed such as (i) development of PVA hydrogels with maximum degree of mineralization keeping the macroporosity and patterning of hydrogels with HA nanoparticles intact, (ii) obtaining the best combination of mechanical properties interns of strength and toughness and (iii) adhesion, proliferation and differentiation of stem cells on mineralized hydrogel's surfaces. Our efforts are on to address the above issues.

4. Conclusions

In summary, the present manuscript is a brief description of a biomimetic method to produce surface functionalized nanocomposite hydrogel scaffold. Such sacffolds may be suitable for bone / cartilage tissue engineering. PVA molecules mediated nucleation and growth of HA nanoparticles and subsequent freeze thawing to induce hydrogen bond *aided* three dimensional physical gelation of hybrid (PVA - HA) system, has been devised to produce nanocomposite scaffolds. An integration of *in situ* mineralization with hydro-gelation could successfully lead to the development of mineralized structure with functionalized surfaces. Manuscript clearly manifested dual role of PVA matrix in the micro-architect of the scaffold. Polymer matrix could successfully control the size of *in situ* precipitated HA nano particles and a systematic variation in PVA concentration (2% to 4%) has manifested a control over the dimensions and geometry of the pores present in the scaffold.

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