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FESTSCHRIFT IN HONOUR OF PROF T R ANANTHARAMAN ON THE OCCASION OF HIS 80TH BIRTHDAY

Morphological changes in biomimetically synthesized hydroxyapatite and silver nanoparticles for medical applications

Tanmay Bera · P. Ramachandrarao

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Abstract A precise control of morphology, which plays a significant role towards the application of materials, can be achieved by studying the effects of macromolecules as nucleation templates for minerals in a biomineralization process. The present investigation aims to understand such effects on medically important materials. Thus, Silver (Ag) and Calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] were synthesized in both biopolymer (gelatin) and synthetic polymer (PVA) media, with aqueous medium as the control to portray a collage of how the interface between the macromolecule and the mineral controls the final morphology of the materials. It was also observed that the change of morphology of the products does influence their performances in specific applications like antibacterial property of the nanoparticles of silver was found to the best when synthesized in aqueous media. Thus, we consider that the organic–inorganic interaction to be of vital significance in the synthesis of complex shapes and sizes of nanoparticles for important applications.

This article is dedicated to Professor T. R. Anantharaman a great teacher and founder of the school of research at the Department of Metallurgical Engineering, Banaras Hindu University (BHU). Interaction with this eminent visionary has greatly influenced our scientific and spiritual thinking.

T. Bera
Department of Metallurgical Engineering, IT-BHU, Varanasi 221005, India
e-mail: tanmay.bera@gmail.com

P. Ramachandrarao (✉)
International Advanced Research Centre for Powder Metallurgy and other New Materials (ARCI), Balapur, Hyderabad 500005, India
e-mail: pramachandra_rao@yahoo.com

Introduction

Natural materials like shells, bones, bamboo etc. are composite materials in which micro- or nano-crystalline materials are systematically distributed in a polymeric matrix [1]. In these natural composites, the remarkable precision with which crystals of identical shapes and sizes are synthesized arises from unique and specific interaction of biological macromolecules with other biomolecules and/or inorganic substances [2]. This precise growth morphology of the inorganic crystals and their distribution in the polymer matrix bestow incredible properties on these materials. Researches have revealed astonishing properties in hither to neglected materials, like cob webs [3], shells [4], or even in mere bacteria [5]. And due to their superior properties these biologically derived materials have become active field of materials research over past few decades. Astonishingly, nature only uses a handful of crystals to make thousands of different varieties of materials, which often are well organized, multi-functional, and structurally hierarchical. Each of these materials is designed for a specific purpose and is morphologically different from others [6]. The chosen organic and inorganic compounds and the interactions that prevail at the interface between them are of paramount importance in designing these natural composites. For example, the shell of an abalone, the mother of pearl, is essentially made of calcium carbonate, but the coin-like crystals of calcium carbonate are sandwiched between the protein layers whose domains absorb the energy under loaded conditions, making it extremely resistant to cracking [7]. Likewise, bone is another nature's wonder materials which is the only tissue that heals completely by itself; thanks to the piezoelectric property [8] of the apatite crystals present in it. The bone, like the shell, is also designed for load-bearing applications. In general, in

these natural composites, the choice of constituents and their systematic arrangement is such that together they serve their application the best way possible. Thus, understanding such structure–property correlation of natural materials can help to achieve great technological breakthroughs [9]. To replicate such material properties for technological applications, it is important to study the natural process of materials' synthesis or biomineralization [6]. Most biomineralization processes take place in chambers, formed by a selectively permeable membrane, containing one or more specific biomolecules. The chambers thus created, hold specific biomolecules inside and allow only the required ions to pass through. The biomolecules are often proteins with specific domains or sequences that can co-ordinate with the incoming inorganic ions. Under such controlled environment, the proteins and/or the macromolecule assume specific conformations to create voids inside them. The macromolecules act as templates for nucleation of the minerals and the process of mineralization continues as a result of several sequential events [10–12].

In our previous report, we mimicked such an environment to explore the importance of semi-permeable membranes during the biomineralization process [13]. In the present investigation we have attempted another simple approach to focus only on the interactions between the organic macro molecule and the inorganic crystals prevalent at their interface to control the morphology of the products. Silver nanoparticles and calcium hydroxyapatite are well known for their use in medical applications. In our previous reports, we have demonstrated the synthesis of stable sols of silver in aqueous media with enhanced antibacterial properties [14]. The nanoparticles of silver were mostly monodispersed and spherical in shape. This enhanced antibacterial effect was brought about by the nanoparticles due to their small size. This helped them to directly interact with the bacterial cell membranes and also with the intra-cellular proteins to cause cell lysis and inhibit certain of cellular mechanisms vital for the bacterial cells to survive. In another of our study, nanocomposites of hydroxyapatite and gelatin were developed for orthopedic applications [15]. It was observed that an appropriate proportion of gelatin and hydroxyapatite is essential for the nanocomposites to have in vitro characteristics similar to the natural bone. A few questions that arose from our earlier studies, pertained to the role of the medium of synthesis, the relative proportion of the medium and the macromolecules in the nucleation phenomena. Another aspect of the problem concerned the dependence of morphology on such variables. To obtain a better understanding of these issues, synthesis of silver nanoparticles and hydroxyapatite were carried out in polyvinyl alcohol (PVA), a synthetic polymer, and in gelatin (an organic polymer); with simple aqueous medium as control. A detailed study was carried out to compare the process of crystallization of these materials in polymeric medium vis-à-vis in simple aqueous solution phase. The whole experiment was designed to simulate the natural process of crystal formation where the inorganic crystals invariably nucleate and grow in a matrix of biopolymer in a precisely controlled fashion.

Having synthesized particles of varied morphologies in different media, we analyzed the changes in their morphology. The nanoparticles showed tendency of aggregation when synthesized in aqueous medium, but the particles synthesized in gelatin medium were found monodispersed and associated with gelatin fibrils. However, an anomaly was observed for silver nanoparticles as the number of particles was really high and slightly smaller in size for the samples synthesized in PVA. The hydroxyapatite particles were found to have distinct morphologies as the growth medium changed. Our investigations on these nanoparticles which are as yet unpublished studies indicated that the antibacterial activity of silver nanoparticles and the biocompatibility and assimilation of hydroxyapatite-based bone substitute depended on the method of synthesis and the resultant morphology of the nanoparticles. For example, when the silver nanoparticles were studied for their antibacterial applications using cultures of *E. coli* as a model of gram negative bacteria, it was found that the antibacterial property was best for the particles synthesized in aqueous medium probably because they were free to interact with the bacteria. The findings collectively revealed that by manipulating the medium of synthesis the organic–inorganic interface can be tailored for producing nanoparticles with appropriate shape and size and of greater utility.

Experimental

Hydroxyapatite synthesis

Calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] was synthesized using calcium nitrate tetra hydrate [$Ca(NO_3)_2 \cdot 4H_2O$] (Qualigens) and di-ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] (S.D. Fine Chem. Ltd.) as precursors for calcium and phosphate, respectively. Initially, three sets of 0.1 (M) solutions of calcium nitrate tetra hydrate were prepared by dissolving 2.36 g in 100 mL of double distilled water (milliQ). Then, 0.25 g each of gelatin (Merck India Ltd) and polyvinyl alcohol (PVA, M.wt 125,000) (S. D. Fine Chem India Ltd) were weighed and separately dissolved in warm solutions of calcium nitrate. This amount of polymer was chosen so that the final proportion of hydroxyapatite and polymer remains 80:20, the approximate proportion of mineral to polymer found in natural bones. The third set, having no polymers, was used as control. The polymer-bearing solutions were aged over night to insure

homogeneous distribution of the calcium ions into the polymer chains. Meanwhile, 0.79 g of di-ammonium hydrogen phosphate was dissolved in dilute ammonia solution to obtain 100 mL of 0.06 (M) solution of phosphate ions having a pH of about 13. Three such sets of phosphate solution were made and then each set was added drop wise to the calcium-bearing polymers solutions (or only aqueous solution) with continuous stirring. On addition of phosphate ions, the solution gradually turns milky white indicating the formation of hydroxyapatite. After the precursor solutions are completely mixed together, the white turbid solutions containing hydroxyapatite nuclei were kept in water bath at 45 °C for 8 h to ensure completion of the crystallization process. The creamy precipitate obtained at the bottom of the vessel, was removed carefully after decantation. Then more distilled water was added to this slurry with stirring and subsequently incubated. The precipitate was again obtained after decantation. This process was repeated twice more to get rid of the soluble by-products. The slurry was then further consolidated by centrifuging it at 1,000*g* for 5 min. It was then dried in air at 40 °C for two complete days to finally obtain bright white looking polymer composites of hydroxyapatite or only hydroxyapatite powders.

Silver nanoparticles synthesis

Silver nanoparticles were synthesized in a fashion similar to the process as described in our previous report [14]. In short, silver nitrate solutions ($AgNO_3$) (Merck India Ltd), mixed with other additives to form a stable soluble complex of silver ions, was dissolved in gelatin and PVA medium separately to obtain 50 mL of 10 m(M) solution of silver ions in 2% polymer. The control study had no polymers in it. The silver ion-bearing solutions were carefully kept in dark for an hour to ensure a proper mixing of silver ions into the polymer. Subsequently, 5 mL of 0.1 (M) D-glucose ($C_6H_{12}O_6$) (Merck India Ltd) solution was injected into each set of the warm (60 °C) silver nitrate solution with vigorous mixing for 15 min. The colors of the solutions changed to dark buff indicating the formation of silver nanoparticles in the solution. The solutions were allowed to cool down to room temperature and then centrifuged at 1,000*g* to get rid of bigger agglomerates. Finally, the silver sols were stored carefully in dark vials or dried in reducing environment to obtain a black powdery mass for further analysis.

Characterization

X-ray diffraction (XRD) studies were carried out using Rigaku D Max III for the samples for the phase identification studies. Fourier transformed infra red spectroscopy (FTIR) (Perkin Elmer) studies were carried out using potassium bromide (KBr) pellets to comment about the inorganic–organic interactions. Ultraviolet (UV) (Pharmacia Biotech) Spectroscopy was carried out to confirm the reduction reaction. Microstructure of the samples was studied by Scanning Electron Microscopy (SEM) (Philips XL-20). Transmission Electron Microscopy (TEM) (Jeol CX 200) was performed to determine the size of the particles.

Antibacterial tests

The dynamic growth rate of *E. coli* (ATCC 25922), as model of gram negative bacteria, was studied under the influence of silver nanoparticles in various medium. The experimental set ups were very similar to the one that we have previously reported [14] but involved slight modifications. About 10^{10} colony forming units of bacteria were inoculated in 100 mL of Luria-Bertani (LB) medium containing 10 ppm of silver nanoparticles dispersed in three different media. The optical density (OD) at 600 nm was assumed as the measure of bacterial growth in the media and the growth curves were obtained by plotting the OD (an average of three readings) with the corresponding time interval over a period of 15 h.

Results and discussions

Phase identification

XRD studies were carried out for phase identification of the samples prepared. The Fig. 1 shows the XRD patterns of

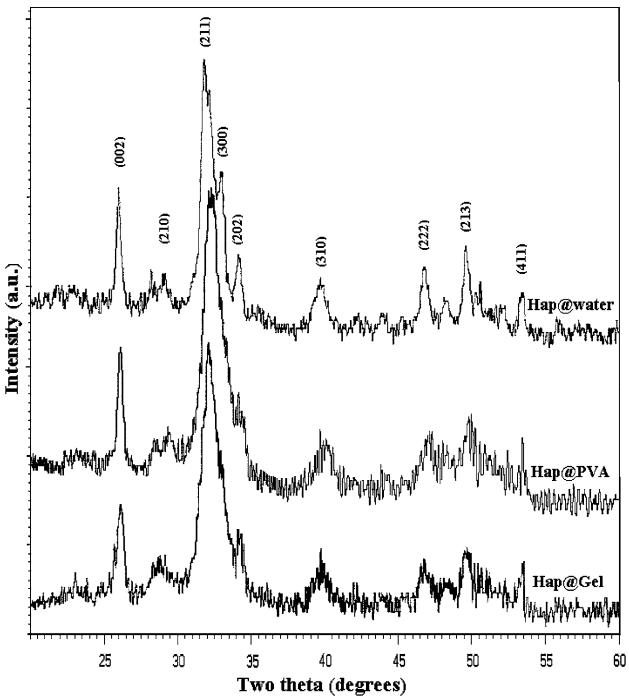

Fig. 1 X-ray diffraction pattern of the hydroxyapatite crystals synthesized in various media

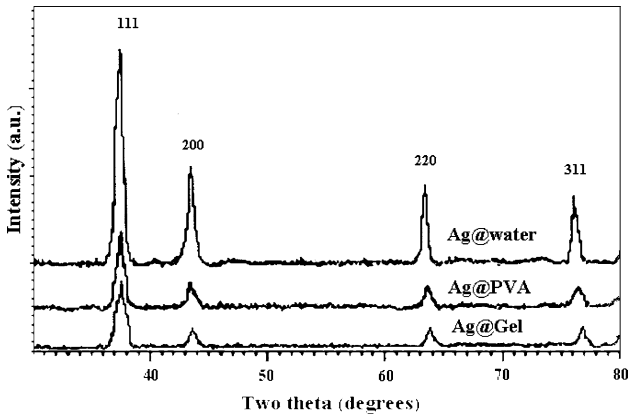

Fig. 2 X-ray diffractogram of the silver nanoparticles formed in various media

the hydroxyapatite samples. The less intensity counts and the broad peaks are due to the poor crystallinity of the hydroxyapatite samples. As evident in the figures the crystallinity was poorest in case of the samples grown in the organic polymer medium of gelatin and a little better in the medium employing a synthetic polymer, PVA; crystallinity was best in the aqueous medium. This is perhaps due to the higher extent of conglomeration of hydroxyapatite particles in the aqueous medium as there are no polymer chains to prevent it from aggregating. Similar kind of patterns (Fig. 2) can be found in case of silver nanoparticles as well. The silver nanoparticles synthesized in gelatin medium show broader diffraction peaks than the particles synthesized in PVA or in aqueous medium. On the whole, XRD results reflect the association of organic molecules with the inorganic phases and their role in maintaining a state of monodispersion thus preventing the crystals from growing freely.

Interactions studies using FTIR

The FTIR spectroscopy was performed to understand the interaction between the inorganic crystals and the organic polymer media. Figure 3 shows the FTIR curves of hydroxyapatite synthesized in different media. Along with it are the curves of gelatin and PVA shown for comparison. The peaks labeled, corresponding to the phosphate group, can be seen shifted for hydroxyapatite samples synthesized in Gelatin and PVA medium from the one formed in aqueous medium. This clearly indicates the interaction between the phosphate group of hydroxyapatite crystals and the polymers in a fashion that occurs in natural bone [16]. Probably, the negatively charged phosphate groups are linked through hydrogen bonds with the positively charged domains of the polymer. Interactions between the calcium ions and the negatively charged functional groups of the polymeric chains like the hydroxyl groups in PVA

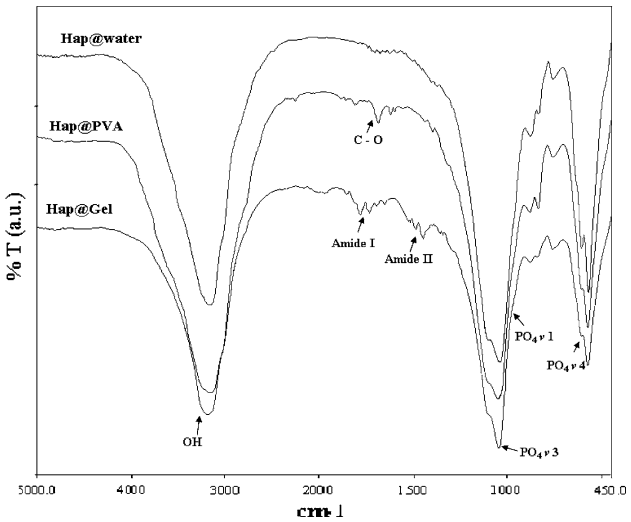

Fig. 3 FTIR spectra of the hydroxyapatite crystals obtained in various media

and also the C terminal in case of gelatin appears to be taking place as there is slight stretching in these groups as well. This interaction of the mineral with the polymer chains were also monitored in the IR spectra of the silver nanoparticles (results not shown). The polymer medium also increases the viscosity of the solution there by helping these particles to remain in suspension discouraging coagulation.

Reduction studies using UV-spectroscopy studies

Ultraviolet (UV) spectroscopy studies were carried out to confirm the reduction of silver nitrate to silver nanoparticles upon the addition of glucose solution. Glucose is an aldehyde which reduces silver ions to silver and oxidize itself to glucolic acid ($C_5H_{11}O_5$–COOH). The reduction of silver nitrate solution by glucose solution can easily be inferred from the spectroscopic results (Fig. 4). The silver

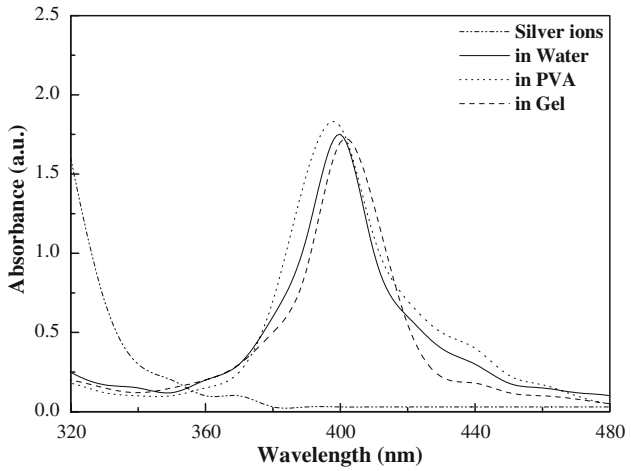

Fig. 4 UV–Visible absorption spectra of the silver nanoparticles

nanoparticles can be identified from the peaks obtained around 400 nm, which is the signature for the silver nanoparticle formation, apart from the color change. Slight shift in the position of the peak can be seen for silver nanoparticles synthesized in different media. This could also be due to the size dependence of the interaction of silver particles with optical radiation resulting in a peak shift. The optical spectra of the silver nanoparticles in the PVA medium show a broader hump suggesting a wider distribution in particle sizes; however, there is a red shift in plasmon of nanoparticles formed in the gelatin medium which probably indicates coarser particles. The plasmon curves also suggest that the size of the mineral formed can be manipulated using the biomimetic principles.

Morphological studies

The samples of hydroxyapatite were studied under the scanning electron microscopes to investigate their morphological characteristics. Figure 5a shows thin plate-like micron sized crystals of hydroxyapatite obtained when the synthesis was carried out in aqueous medium. This is possibly due to freer crystallization in the aqueous medium. It is presumed that the absence of polymer chains allowed crystals to grow continuously along the direction opposite to the heat flux. Very similar features were obtained for the apatite crystals formed in the PVA media (Fig. 5b). However, the plates of the crystals were found to be smaller and intricately associated with the polymer molecules that cover them completely. The presence of these polymer molecules can hinder their growth to bigger dimensions. However, as shown in Fig. 5c, the microstructural features are not revealed, even at higher magnification when the synthesis was carried out in gelatin medium. This is due to the smaller sizes of the hydroxyapatite crystals formed in this case which appeared as rather single phase. As shown in transmission electron microscopic study (Fig. 5d), the hydroxyapatite crystals formed in gelatin are nano-sized needles that are also tangled in the gelatin mesh. The selected area electron diffraction pattern shows smooth concentric rings indicating the fineness of the crystals and their random orientation in the composite.

The particles of silver show similar diversities in their morphologies when synthesized in different media (Fig. 6). The particles which were synthesized in aqueous media (Fig. 6a) show mostly spherical particles about 20–25 nm in diameter (Fig. 6a). Aggregates of the particles mostly appear to arise from flocculation. This can be due to the higher reaction kinetics and the absence of any polymer stabilizer. The particles formed in the PVA medium are of larger number which probably explains the darker color of the solution. The particles are of several sizes, but mostly polyhedral having several facets (Fig. 6b). The particle size

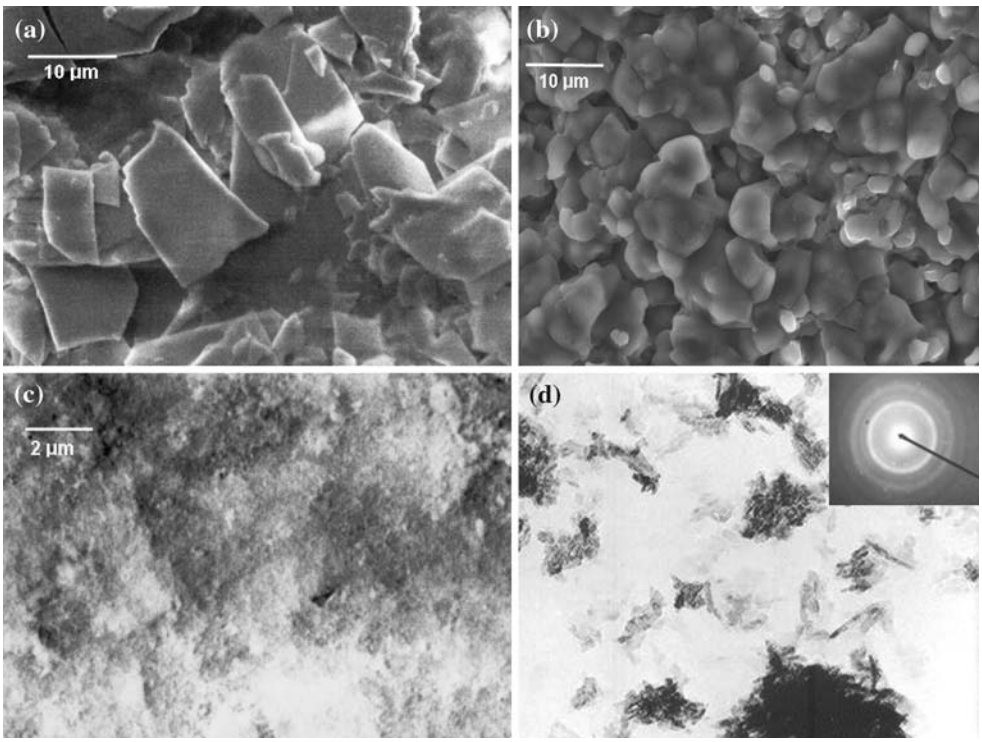

Fig. 5 Microstructure of the hydroxyapatite crystals synthesized (**a**) in water (**b**) in PVA and (**c**) in gelatin (**d**) TEM microphotograph of hydroxyapatite crystals formed in gelatin medium at 29 K magnification

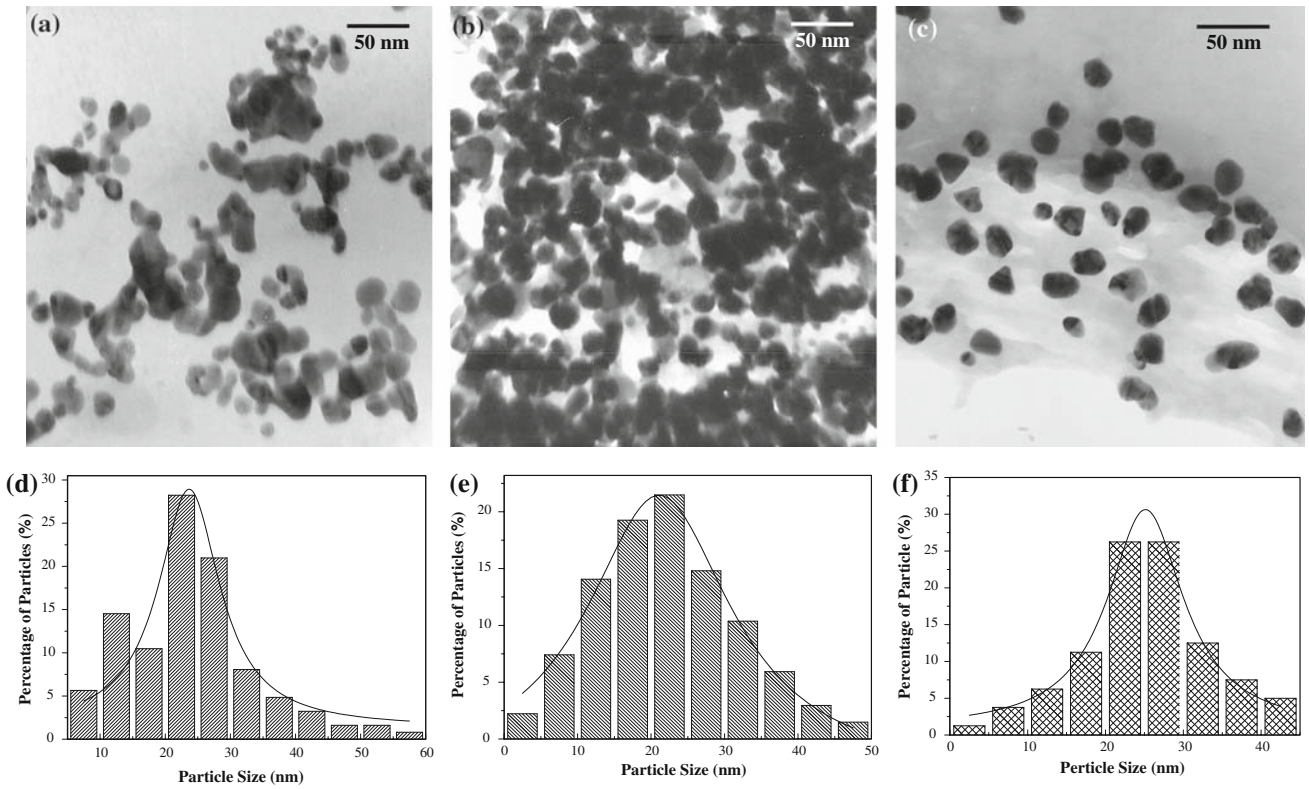

Fig. 6 TEM microphotograph of Silver nanoparticles (**a**) in water (**b**) in PVA and (**c**) in gelatin and (**d–f**) showing the particle size analysis of the particles (in the same order)

distribution is also wider in this case. This is taken to imply less control on kinetics. It seems that PVA, being a poly-ol, also acts a reducing agent at this reaction temperature enhancing reduction kinetics [17]. The presence of two reducing agents glucose and PVA aggravates the reduction kinetics. Due to this more nuclei of silver are formed in this case leading to the formation of higher number of particles and eventually a higher concentration of particles in the media. However, the particles maintain a better state of dispersion than those in aqueous medium probably due to the presence of polymer stabilizers. The particles formed in the gelatin medium on the contrary are bigger in size, slightly anisotropic, and mono-dispersed. Gelatin, in spite of not being a polyelectrolyte itself, manages to interact with metal nanoparticles and serves as a capping agent, due to its mesh-like structure rather than any particular functional group. Silver ions have a good affinity to proteins particularly to the peptide residues with thiol and disulphide groups. Hence the gelatin allows the tiny silver nuclei to grow slowly to become bigger particles without fusing to another one and also helps them remain monodispersed in the medium.

The morphological changes along with the other features strongly suggest that the growth morphology of inorganic crystals is largely influenced by the organic macromolecules that are present during their formation. This can be attributed to the chemistry at the inorganic organic interface during the nucleation and growth of the particles. In solution the polymer molecules get dispersed and orient themselves favorably according to the charges distributed on them. This allows them to interact with the inorganic ions like that of calcium and silver [18]. PVA is basically a single chain poly-ol which is rich in hydroxyl (OH) groups. These OH groups however undergo both inter- and intra-molecular hydrogen bonding making PVA solution viscous. The lone pairs of electrons of the OH groups that do not participate in such hydrogen bonding can easily co-ordinate with the metal ions which often have high charge density and vacant *d*-orbitals (for transition metals like silver). When such co-ordination takes place between one metal ion and several adjacent OH groups the metal ion gets covered with PVA chains. Similar mechanisms also operate in gelatin as it is rich in functional groups like COO, CHO, CO, OH, SH, S–S. Gelatin, being a denatured protein, exhibits both the primary and a secondary structure which is a gel-like fibrous structure. This helps it to completely cover or engulf the metal ion from all sides something that PVA fails to do appreciably due to its linear structure and smaller molecular size. Due to the total engulfment of metal ions by the gelatin molecule, the inorganic mineral formed in the molecular cavity of gelatin is smaller in size, similar shape, and also monodispersed in state; something very similar to the natural process of biomineralization.

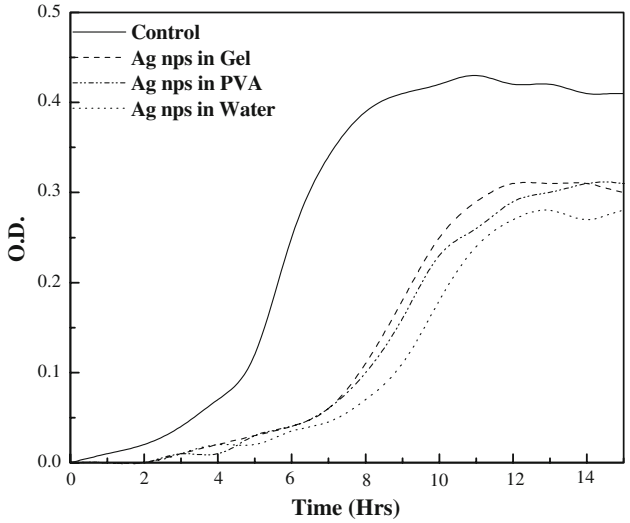

Fig. 7 Antibacterial activity curves for silver nanoparticles

Antibacterial study

To evaluate the antibacterial effects, the dynamic growth rates of *E. coli* were monitored in culture media containing only 10 ppm of silver nanoparticles formed in three different media. The presence of nanoparticles retarded the growth rate of the bacteria in all cases almost to the same extent (Fig. 7). However, as seen from the figure the particles synthesized in aqueous media showed the antibacterial property and the one synthesized in the PVA slightly better than that obtained in gelatin media. It was found from our previous studies that the nanoparticles of silver needs to interact with the bacterial cell to bring about the antibacterial effect. Hence, the lower antibacterial effects of the nanoparticles, synthesized in the polymer media, can be attributed to their intimate association with the polymer chains making them less available to the bacterial interaction therefore causing slightly lesser antibacterial effect.

Conclusion

Medically important materials like hydroxyapatite and silver nanoparticles could successfully be synthesized in both gelatin and PVA media and also in simple aqueous medium employed as the control. There was a difference in size and shape of the particles grown in different media. The differences arise from varied nature and extent of interaction between the polymer domains and the specific growth planes of the inorganic particles. The properties of the material also change by such an interaction. The nanoparticles of silver grown simply in aqueous media were found to be more potent in antibacterial applications. Therefore, by understanding the underlying mechanism of biomineralization, it is possible to manipulate the morphology of the particles and also improve their performance. The present study is a small step towards understanding the interaction between the interfaces of the biomolecules and the inorganic crystal. Further studies are needed to probe the detailed growth mechanism of crystallization during mineralization process following the biomimetic principles which will empower us with a better and safer technology.

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