Bull. Mater. Sci., Vol. 36, No. 7, December 2013, pp. 1165–1170. © Indian Academy of Sciences.

# Iron nanoparticles from blood coated with collagen as a matrix for synthesis of nanohydroxyapatite

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MS received 27 April 2012; revised 9 September 2012

**Abstract.** **A simple wet precipitation technique was used to prepare nanobiocomposite containing iron nanoparticles coated with collagen. This nanobiocomposite was used as matrix for the synthesis of nanohydroxyapatite. The physicochemical characteristic studies of the nanohydroxyapatite thus formed were carried out using fourier transform infrared spectroscopy, transmission electron microscopy, scanning electron microscopy, energy-dispersive X-ray spectroscopy and X-ray diffraction technique to confirm the formation of hydroxyapatite on iron nanoparticle–collagen complex. The results of the above studies supported the formation of iron nanoparticle–collagen–hydroxyapatite composite. The biological studies such as biocompatibility and hemocompatibility were carried out for nanohydroxyapatite using different cell lines and blood sample. The results of biocompatibility and hemolytic assay revealed that the prepared nanobiocomposite was 100 % biocompatible and hemocompatible. This nanobiocomposite may be used for biomedical application such as injectables for targeted delivery and as scaffold for tissue engineering.**

**Keywords.** **Collagen; iron nanoparticle; nanohydroxyapatite; MTT assay; wet precipitation method.**

## 1. Introduction

Bone is a type of connective tissue mainly composed of an organic component, collagen and an inorganic component, nanocrystalline carbonate hydroxyapatite. This type of biological hybrid material finds potential biomedical application in bone tissue engineering by promoting the osteoblast proliferation and differentiation. It is also extensively used in orthopaedic surgery as bone-filling material (Maria and Daniel 2006; Tsai *et al* 2008). Nanotechnology has gained extensive biomedical applicability in various fields such as nanobiomaterials, nanoparticles (Nps) for the targeted medicine and gene therapy. Among the available inorganic biomaterials, nanohydroxyapatite (*n*HAp) is widely used as filler for bone disorders due to its good biocompatibility and osteoconductivity. Apart from its use as bone substitute, it can also be used as a carrier for chemotherapeutic agents such as doxorubicin, mitomycin C and 5-fluorouracil in cancer treatment (Wang *et al* 2009). The surface of polymer components containing polyvinyl alcohol–polyvinyl pyrrolidone (PVA–PVP) is chemically multifunctional (surface OH, COOH, C=C, C–O–C and C=O groups exist). HAp can be easily grown on such polymer surfaces by two major phenomena such as physical adsorption and chemical interaction. Natural biopolymers such as collagen, fibrin, alginate and hyaluronic acid can be used apart from the commercially available synthetic polymers. The precipitation of HAp crystals on polymers can be carried out under mild temperature conditions (Geiger *et al* 2003; Oudadesse *et al* 2011). Apart from polymers, the biomedical materials such as metals, ceramics or hybrid combinations can be used for preparing the biomimetic nanocomposites (Tan and Saltzman 2004). The cell viability, attachment and proliferation studies using cell lines were carried out to check the cytocompatibility nature of the nanocomposite scaffolds. Cell lines such as osteosarcoma (MG-63), normal vero cell lines, mouse embryonic fibroblast cells (NIH3T3) and normal human dermal fibroblast (*n*HDF) cells are widely used for such purposes. The cell line studies suggest that the prepared material could be a potential candidate for bone and wound tissue engineering purpose (Sudheesh Kumar *et al* 2011).

Collagen, a biopolymer finds its application in tissue engineering including skin replacement, bone substitutes, artificial blood vessels and valves. Collagen is available in various forms for different applications such as collagen shields in ophthalmology, sponges for burns/wounds, minipellets and tablets for protein delivery, gel formulation in combination with liposome's for sustained drug delivery, as controlling material for transdermal delivery, as ophthalmic inserts, Nps for gene delivery and basic matrices for cell culture systems (Lee *et al* 2001; Mu *et al* 2003; Ruszczaka and Friess 2003;

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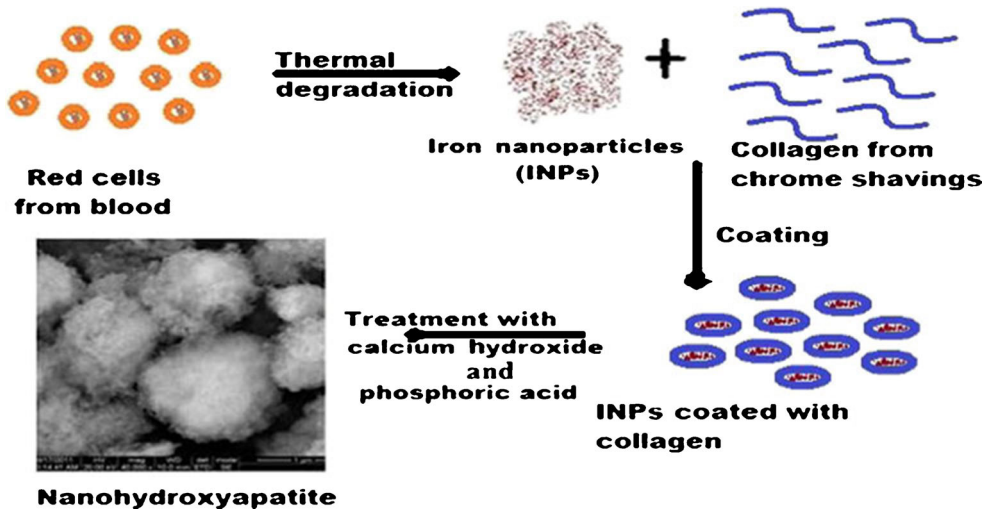


**Scheme 1.** Graphical representation for preparation and characterization of INP–collagen–HAp.

Huang *et al* 2009; Nair *et al* 2010). Chromium-containing leather wastes (CCLW) from post-tanning operations have the potential to generate value-added material like collagen. By alkali and enzymatic hydrolysis of CCLW, various useful products such as gelatin, collagen and chromium-containing retanning agents for leather industry are produced (Mu *et al* 2003). Hyperthermia, a thermal therapy for cancer has drawn more attention for *in vivo* cancer treatment. Such treatment is possible with the new magnetic hydroxyapatite nanoparticles which were first made by co-precipitation method (Hou *et al* 2009).

The biocompatible iron oxide nanoparticles (INPS) find their applications in various fields such as biology, medical diagnostics and therapy. Some of the biomedical applications include targeted drug delivery, diagnostics, biosensors, MRI contrast agents and hyperthermia (Morteza *et al* 2011). Magnetic scaffolds containing INPS are promising for magnetic guiding in orthopaedic tissue engineering applications and they are suitable to treat several pathologies in regenerative medicine (Panseri *et al* 2012). Using computer simulations, INPs incorporated hydroxyapatite scaffolds help in investigating the osteogenesis with the guidance of the magnetic field, they generate (Bock *et al* 2010). So, by considering the wide applicability of INPs, *n*HAp and collagen as mentioned above, a collagen based metal nanobiocomposite was synthesized in this study, which may act as inorganic biomaterial for bone substitute, as a nanocarrier for chemotherapeutic agent and as hyperthermia agent due to the presence of iron nanoparticles (INPs). The novelty of this study lies in the raw materials used for the preparation of *n*HAp. The raw materials such as collagen and INPs used for the preparation of nanobiocomposite were isolated from biological waste materials such as CCLW and goat blood from municipal slaughter house. The graphical representation for the preparation and characterization of *n*HAp is given below (scheme 1).

## 2. Materials and method

### 2.1 *Materials*

Goat blood was collected from a nearby municipal slaughter house, CCLW was collected from the Department of Tannery, Central Leather Research Institute, Chennai; MTT (3-(4, 5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide) and calcium hydroxide were purchased from Merck Ltd, India and all other chemicals used were of analytical grade. MG-63, NIH3T3, epidermoid carcinoma (A431) and normal Vero cell lines were obtained from the National Centre for Cell Science (NCSS), Pune.

### 2.2 *Preparation of INPs and collagen from natural waste*

INPs from animal blood was prepared as reported in our earlier studies (Chamundeeswari *et al* 2011) and briefly elaborated as follows: 1 L of goat blood was collected and stirred mechanically using a glass rod for 15 min continuously to isolate the fibrin. The defibrinated blood was centrifuged at 7155 g for 20 min; the supernatant (serum portion) was discarded and the red blood cells (RBC) collected at the bottom of the tube was removed, washed with water for 10 times and stored at 4 °C. RBC was then incinerated in a silica crucible using a muffle furnace at 800 °C for 2 h. After cooling, the residue (INPs) was collected and stored in a glass container. The collagen was prepared by dechroming CCLW using sulphuric acid. The dechroming was done as follows: CCLW was soaked in water for 24 h. Later the water was decanted and treated with 0·5 N sulphuric acid for 5 h and checked for the dechroming (CCLW lot originally exhibits blue colour whereas the dechromed lot shows brown colour). The dechromed sample was then treated with

0·1 M tris HCl (pH 8), 0·2 M $\beta$-mercaptoethanol, 0·05 M EDTA for 3 days to obtain collagen in fibre form. The collagen fibrils were suspended in 0·05 M acetic acid and digested with 19 % 100:10: dechromed tissue: pepsin at 4 °C for 24 h. The pepsin solubilized collagen was centrifuged at 7155 g for an hour. The supernatant was further dissolved in 0·05 M acetic acid and subsequently dialyzed with water and the obtained collagen was stored for further use and analyses.

### 2.3 *Synthesis of nanohydroxyapatite (nHAp)*

*n*HAp was prepared by wet precipitation technique. 40 mg of INPs was weighed and dissolved in 600 $\mu$L of phosphoric acid. To this, 10 mL of water and 6 mL of 2 % collagen solution, isolated from CCLW was added and labelled as mixture 1. The mixture 1 was allowed to stand for 15 min. To 1·5 g of calcium hydroxide, 5 mL of water and 400 $\mu$L of 4 % ethanol were added and the solution was labelled as mixture 2. The mixture 2 was then taken in a 100 mL beaker and kept for stirring at 30 °C. To this, 16 mL of mixture 1 was added drop wise and after addition, pH of the resultant solution was found to be 14. pH was then adjusted to 10 using dilute HCl followed by stirring for 1 h and the resulting precipitate was kept for aging at room temperature (30 °C). The precipitate formed was centrifuged, washed repeatedly with water and the final precipitate (*n*HAp) collected after thorough washing and dried at 100 °C for 5 h.

### 2.4 *Characterization*

Fourier transform infrared (FT–IR) spectra of the prepared samples were recorded on a Nicolet 360 FT–IR spectroscope using KBr pellet containing 2–6 mg of sample to confirm the presence of HA on collagen. The surface morphology of the composite was visualized by scanning electron microscope (SEM) and elemental analysis was carried out using energy–dispersive X-ray (EDX) spectroscopy. The sample was placed over the aluminum stab containing carbon sheet and air dried for a few minutes. Then the dried specimen was sputter coated with Au and analysed under the following condition: 0·1 Torr pressure, 200 Ma current and 70 s coating time. The surface morphology was visualized by SEM (Hitachi SEM; S-3400N model) using 10 kV as an accelerating voltage. The elemental analysis to estimate the amount of calcium phosphate ratio was carried out using EDX spectroscopy (Thermo EDX) at an accelerating voltage of 10 kV. The transmission electron microscopy (TEM) analysis for the prepared sample was carried out using Tecnai 10, Philips TEM to determine the size and shape of the prepared *n*HAp. The sample was sonicated using Vibronics, Ultrasonicator processor 2 at 180 Watt for 20 cycles, each cycle for 1 min with a gap of 30 s after each cycle. These particles were then mixed with 2% phosphotungstic acid with a ratio of 1:2 and 20 $\mu$L of the mixture was placed in copper grid 260 # and dried at room temperature for analysis. X-ray diffraction (XRD) analysis of the sample was done using X-ray diffractor GE model 3003TT German, as a further confirmatory study for the formation of *n*HAp on collagen.

### 2.5 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis*

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out using Laemmli's method and 8% gel was prepared. The sample was dissolved in SDS sample buffer solution containing 1% SDS, 1% mercaptoethanol and 20% glycerol and heated for 5 min at 100 °C. The sample 40 $\mu$L (100 $\mu$g) was subjected to gel electrophoresis at a constant current of 5 V/cm. After electrophoresis, the gel was stained with 0·1% coomassie brilliant blue R-250 and the gel images were captured on a BIOVIS gel documentation system.

### 2.6 *Cell culturing studies*

The biocompatibility test for the prepared *n*HAp was evaluated using MTT assay. The cell lines used to check the biocompatibility are MG-63, A431, normal vero cell line and NIH3T3. They are cultured in $\alpha$-MEM (MG-63) and DMEM (NIH3T3, A431 and normal vero cell line) supplemented with 10% FBS, 100 IU/mL penicillin, 100 $\mu$g/mL streptomycin, 30 $\mu$g/mL gentamycin, 0·2 $\mu$g/mL fungizone and 2·5 $\mu$g/mL dimethyl sulfoxide (DMSO). The *n*HAp was UV sterilized for 2 h and varying concentrations of the sterilized *n*HAp 25, 50, 75 and 100 $\mu$g were added to 48 wells plate at a seeding cell density of $12 \times 10^3$ cells/well and incubated for 24 h at 37 °C in humidified atmosphere (5% $CO_2$ in 95 % air). After incubation period MTT was added, incubated for 4 h and checked for absorbance at 630 nm using microtitre well plate spectrophotometer (Bio-Rad 680, USA) (Sudheesh Kumar *et al* 2011).

### 2.7 *Phase contrast microscopy studies*

5 mL of 0·5 mg/mL INP–collagen–HAp suspension was prepared using sterilized double-distilled water. The sample was sonicated and the suspension was then sterilized by exposing it to UV radiation for 2 h. MG-63 and normal Vero cell lines were used to check the morphological changes of the cells after treatment with *n*HAp suspension, which may be used as an injectable material. Varying volumes of suspension containing various concentrations of *n*HAp: 5, 12·5 and 25 $\mu$g for MG-63 and 62·5, 125 and 500 $\mu$g for normal Vero cell lines were added to visualize the morphological compatibility with the prepared *n*HAp suspension.

### 2.8 *Hemolytic test*

1 mL of human blood was collected from a healthy 27 year old volunteer (male). The collected blood was diluted with

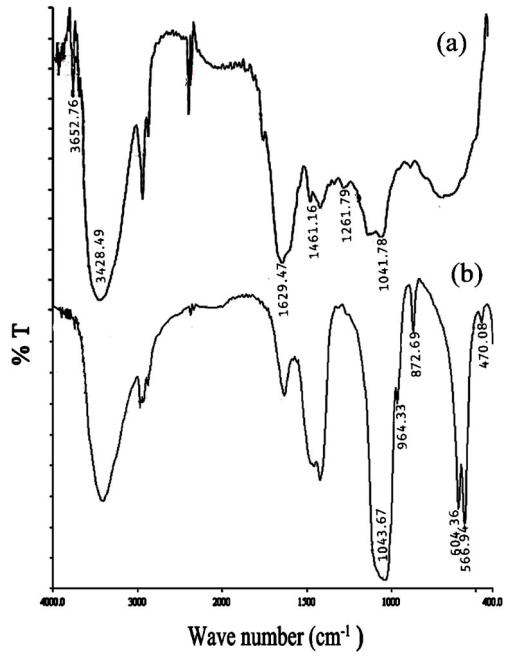


**Figure 1.** (**a**) FT–IR spectrum of collagen isolated from CCLW and (**b**) *n*HAp formed in INP–collagen complex.

10 mL of 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer (pH 7·4). Then the diluted blood was centrifuged at 1431 g for 10 min at 4 °C. After centrifugation, a buffy coat layer was formed in the supernatant and layer formed was removed using a glass rod. This step was repeated thrice and then the final volume of the solution was adjusted to 10 mL after complete removal of the buffy coat. Varying volumes of the diluted blood: 5, 10, 20, 30 and 40 $\mu$L were taken in microfuge tubes, volume of the solution was then adjusted to 1mL using distilled water and centrifuged at 1431 g for 10 min. Then the supernatant was measured at 540 nm using spectrophotometer. The microfuge tube containing 40 $\mu$l of diluted blood showing an OD value of 0·24 was selected, which shows a high hemoglobulin content. This particular volume was selected to check the hemocompatibility of the prepared *n*HAp. 40 $\mu$L of diluted blood was added to the microfuge tubes, containing varying concentrations of *n*HAp: 25, 50, 75 and 100 $\mu$g of *n*HAp and the volume was adjusted to 1ml using HEPES buffer. Two microfuge tubes, one without *n*HAp (positive control) and another with HEPES buffer alone (negative control) were used as controls for this experiment. The above tubes were incubated at 37 °C for 30 min and centrifuged at 1431 g for 10 min. Then, the microfuge tubes were viewed for free hemoglobin in the supernatant (Li *et al* 2009).

## 3. Results and discussion

FT–IR spectra (figure 1(a, b)) of *n*HAp prepared using collagen as matrix shows characteristic absorption bands of both collagen and HAp. The characteristic amides I, II and III absorption bands of collagen (Figure 1(a)) were found at 1629, 1585 and 1241 $cm^{-1}$, respectively. The hydroxyl groups present in the collagen molecules are seen as a broad peak from 3500–3100 $cm^{-1}$ (Palpandi *et al* 2010). The vibration modes of $\nu 1$, $\nu 3$ and $\nu 4$ of the phosphate ions in *n*HAp (figure 1(b)) were also observed. $\nu 3$ band was observed around 1043 $cm^{-1}$ and $\nu 4$ vibration modes were observed at 604, 566 and 470 $cm^{-1}$. The peak at 872 $cm^{-1}$ shows presence of $HPO_4^{-3}$ ions and a band at 964 $cm^{-1}$ represents $\nu 1$ stretching modes of the phosphate. These bands and their positions in FT–IR spectrum indicate the formation of *n*HAp on INP–collagen complex (Sastry *et al* 2008; Chamundeeswari *et al* 2010). XRD diffraction peaks at 31·7, 45·3 and 56·2° indicate reflections from 112, 222 and 004 crystal planes, respectively (see ESI). These reflections more or less correspond to the Bragg reflections of *n*HAp (Tsai *et al* 2008; Ko *et al* 2011). These results show that *n*HAp was formed on INP–collagen complex. SEM images (see ESI) of the individual and aggregate particles of the samples show that they are spherical in shape with a particle size in the range of 6–19 nm. However, the aggregated particle size was found to be 19 nm and they resemble flower-like porous crystalline structure. EDX spectrum (see ESI) has shown presence of calcium and phosphorus in the composite with a ratio of 1·64:1, which is nearer to that of *n*HAp (Sundaraseelan and Sastry 2007). The presence of Fe indicates that the composite formed was metal nanobiocomposite (INP–collagen-HAp). TEM studies (see ESI) revealed shape and size of the nanobiocomposite particles. The particle shape varies from spherical to ovoid with size in the range of 6–19 nm. Due to the aggregation of Nps, some of the particles are seen to be large and TEM micrograph clearly exhibits crystalline nature of the sample (Sanosh *et al* 2009). SDS PAGE results (see ESI) revealed that the isolated biopolymer from CCLW was type I collagen by displaying one $\beta$ band and two $\alpha$ bands ($\alpha_1$ and $\alpha_2$) which were unfolding polypeptide chains of the triple helix. These patterns were similar to the collagen isolated from the cat fish (*Tachysurus maculatus*) by pepsin digestion (Bama *et al* 2010). MTT assay is an *in vitro* analysis, which is used to check biocompatibility of the prepared biomaterial. This assay is a pre-requisite to carry out *in vivo* analysis and also elaborates the biological/cellular responses such as cell viability, attachment and proliferation, alkaline phosphatase activity and osteocalcin formation. In the present work, *in vitro* study is carried out using both osteoblast and fibroblast cell lines such as MG 63, NIH3T3, *n*HDF (human dermal fibroblast) and murine fibroblast L929. This study exhibits a high biocompatibility and adhesion property, which has been reported in earlier works (Zhang *et al* 2003; Nabakumar *et al* 2009; Sudheesh Kumar *et al* 2011). MG 63 which is widely used cell line for MTT assay of HAp is employed in this study (Zhang and Zhang 2004; Sohn *et al* 2006; Teixeira *et al* 2007). MTT assay carried out for *n*HAp/collagen/alginate composite (Zhang *et al* 2003), *n*HAp scaffolds (Teixeira *et al* 2007), HAp/chitosan phosphate nanobiocomposite (Nabakumar *et al* 2009), anodized titanium coated with

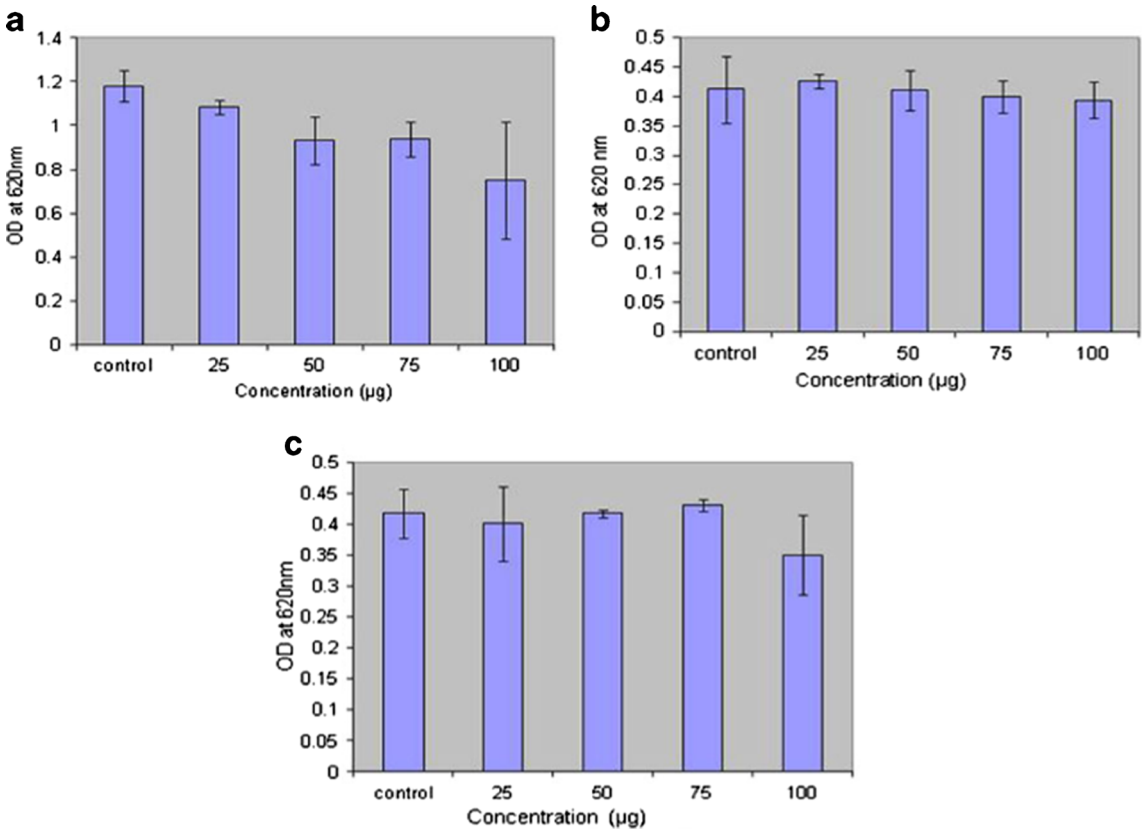


**Figure 2.** MTT assays of normal and cancer cell lines (**a**) mouse embryonic fibroblast cells (NIH3T3), (**b**) osteosarcoma (MG-63) cells and (**c**) epidermoid carcinoma (A431) cells treated with varying concentrations of *n*HAp: 25–100 $\mu$g/well containing $12 \times 10^3$ cells.

HA (Sohn *et al* 2006), $\alpha$-chitin hydrogel/*n*HAp composite scaffold (Sudheesh Kumar *et al* 2011) and biomimetic *n*HAp/polymer matrix such as PVP and PVA (Oudadesse *et al* 2011) exhibited high biocompatibility. Similarly in our study, MTT assay was carried out using cell lines such as NIH3T3 (figure 2(a)), MG 63 (figure 2(b)) and A431 (figure 2(c)) with varying concentrations of *n*HAp composite. 25–75 $\mu$g of *n*HAp shows high viability rates that are comparable with the control. In the case of MG 63 cell lines treated with 100 $\mu$g of *n*HAp shows slight decrease in viability rate when compared to control and this may be due to overloading of *n*HAp to cell lines. The high viability exhibited by *n*HAp treated normal and A431 cells support that the prepared *n*HAp is biocompatible and can be used for various biomedical applications. The magnetic nanoparticles (MNPs) in the form of maghemite can be used for biological applications such as MRI contrast agent, cell labelling and tracking. This type of MNPs exhibited good biocompatibility (Yajie *et al* 2011; Mornet *et al* 2002) and comparatively similar result has been obtained in our study for INPs (maghemite) coated with collagen being used as a matrix for the growth of *n*HAp. Thus prepared novel *n*HAp may also be tried as a scaffold in tissue engineering as an osteoinductive material and as nanocarrier for chemotherapeutic agents (Mateus *et al* 2007; Wang *et al* 2009).

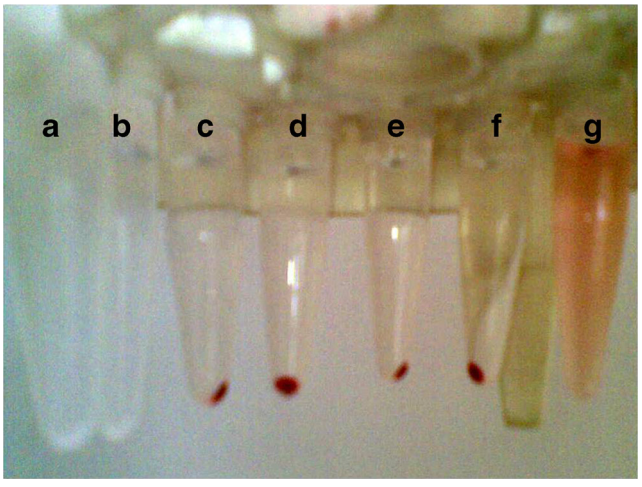


**Figure 3.** Visual observation of hemolytic tests (**a**, **b**) positive controls treated with buffer solution alone, (**c**–**f**) varying concentration of *n*HAp (25–100 $\mu$g) treated red cells showing absence of hemolysis and (**g**) negative control containing water and red cells which shows the presence of hemolysis.

Phase contrast microscopy observations (see ESI) of *n*HAp treated MG-63 and normal Vero cell lines revealed a high density live adherent cells similar to that of control

cells (untreated) even after 24 h incubation (Hua *et al* 2008; Peng *et al* 2011). The visual observation of hemolytic test (figure 3) shows absence of red cell lysis in positive control and *n*HAp (25–100 $\mu$g) treated samples, but hemolysis was observed in negative control. The absence of hemolysis in treated cells proves good hemocompatibility and suggests that the prepared nanobiocomposite may be used as an effective biomedical material that might have clinical applications, in particular, as substrates for tissue engineering purposes and as nanocarrier for chemotherapeutic agents.

## 4. Conclusions

*n*HAp was prepared using INP-collagen as a matrix. The physiochemical studies have proved the synthesis of *n*HAp and the cell culture studies revealed that the nanobiocomposite might be tried as a carrier for chemotherapeutic agents in targeted delivery system.

## Acknowledgments

The authors would like to thank Mr Sekar, Research Associate, CLRI, Chennai, for helping in the preparation of collagen from CCLW, Mr Venkat and Ms Rachita, Biomaterials Division CLRI, Chennai, for helping in cell line studies, Mr Sridharan and Hemachandran for preparing graphical abstract and Khemlal Adikarai, Technical Assistant, St Joseph's College of Engineering, Chennai, for helping in the collection of goat blood.

## Electronic Supplementary Material

Electronic supplementary material pertaining to this article is available on the Bulletin of Materials Science website (www.ias.ac.in/matersci).

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