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Tryptophan complexed hydroxyapatite nanoparticles for immunoglobulin adsorption

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Abstract The selective removal of immunoglobulin using different affinity–type particulate adsorbents has clinical significance in certain autoimmune diseases. This study reports the use of modified nanosized hydroxyapatite as a matrix for affinity based immunoglobulin adsorption. The adsorbent matrix consists of cyclodextrin complexed hydroxyapatite nanoparticles modified with tryptophan. It appears that presence of cyclodextrin has a synergic effect in the adsorption of immunoglobulin proteins having affinity with tryptophan complexed hydroxyapatite. The complexes were analyzed by X-ray diffraction (XRD), transmission electron microscopy (TEM), Fourier transform infrared (FT-IR) spectroscopy, thermal gravimetric analysis (TG) and differential scanning analysis (DSC) methods. The preferential affinity of the immunoglobulin towards tryptophan complexed particles was confirmed with polyacrylamide gel electrophoresis (PAGE) and Lowry adsorption techniques. Immunoglobulin adsorption was confirmed by quantitative turbidimetric assay using a standard immunoglobulin kit. The cytotoxicity index of the nanoparticle complexes were evaluated by MTT assay. Our proposed matrix for immunoglobulin adsorption is cost effective and adaptable for applications towards plasma perfusion.

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1 Introduction

The growing use of nanoparticles in clinical diagnostic processes has set a new trend towards the simplification and improvement of their analytical methods [1]. Serum, that contains thousands of proteins can often serve as an indicator of diseases and is a rich source for biomarker discovery [2]. However the versatile and more abundant range of proteins present in serum masks the detection and determination of the low-abundance proteins that are potential biomarkers for various diseases. Thus methods for the fractionation, isolation, and separation of abundant proteins like albumin and immunoglobulin from human serum are imperative for diagnostic purposes [3, 4]. Further the selective removal of immunoglobulin using different affinity–type particulate adsorbents has clinical significance in certain autoimmune diseases [5].

Classical procedures used for the separation of immunoglobulin like affinity and protein A chromatography have contamination and denaturing problems and are unsuitable for therapeutic purposes [6]. These limitations have necessitated the development of other separation techniques, and have prompted researchers to focus their efforts on the development of new adsorbents with affinity, specificity, and selectivity enabling the separation of immunoglobulin under mild conditions to preserve its integrity and activity. Pseudo bioaffinity ligands on a suitable matrix used to separate several proteins are stable, simpler, less expensive and extremely versatile because of the presence of several molecular interactions [7–9]. Various affinity-type micro particulate adsorbent matrices and affinity columns based on the principle of immobilized metal–chelate affinity chromatography have also been used for the selective removal of the immunoglobulin class of antibodies from human plasma [10]. Hydroxyapatite

($Ca_{10}(PO_4)_6(OH)_2$), a hydrated calcium phosphate is one of the most extensively employed calcium phosphates owing to its similarity to the main mineral constituent of bone tissue [11]. The hydroxyl ions in the hydroxyapatite crystal play an important role in ion exchange, solubility and surface modification [12]. Hydroxyapatite crystals have been frequently employed with Protein A affinity chromatography for the purification of therapeutic antibodies [13]. A number of studies have also depicted the sensitivity of adhered proteins and subsequent cellular behaviour to surface hydrophobicity induced onto hydroxyapatite [14]. Hydroxyapatite has also been utilised for the one-step purification of poly(His)-tagged recombinant protein and is widely used as a promising adsorbent for immobilized column chromatographic techniques [15, 16]. Our group has earlier reported an affinity immunoadsorbent based on hydroxyapatite microspheres for direct hemoperfusion applications [17].

In the last decade numerous studies involving nanoparticles for their applications in separation and purification of proteins and cells are available in literature. Since nanoparticles provide increased surface area available for attachment of ligands they can also be preferentially utilized for specific adsorption. This present study thus focuses on the use of modified nanosized hydroxyapatite as a matrix for affinity based immunoglobulin adsorption. Our proposed matrix can be used for affinity immunoadsorbent suitable for extracorporeal perfusion of plasma for selective removal of antibodies belonging to immunoglobulin G class. The most important advantage of adsorption plasma therapy over plasma exchange are the selective removal of pathogens without loss of essential plasma components.

There have been other studies reporting the synthesis and application of hydroxyapatite nanoparticles in protein adsorption available in literature [18]. In our previous work we have evaluated the use of cyclodextrin complexed hydroxyapatite nanoparticles for albumin adsorption [19]. Our present study focuses on the incorporation of tryptophan into cyclodextrin complexed hydroxyapatite nanoparticles, its effects on the phase of the parent complex and their responses to proteins and immunoglobulin. The nanoparticle complexes were characterised for phase analysis, size distribution, protein adsorption studies and cytotoxicity. The phase analysis of the complexes was carried out by X-ray diffraction and Fourier transform infrared spectroscopy. The complexation was analyzed by Thermal Gravimetric analysis and Differential Scanning Colorimetric studies. The final particle size of the nanoparticle complexes was estimated by Dynamic light scattering and transmission electron microscopy techniques. Protein adsorption on the nanoparticle complexes was studied by Polyacrylamide gel electrophoresis and substantiated by Lowry methods. Immunoglobulin adsorption was evaluated by quantitative turbidimetric assay using a standard immunoglobulin kit. The cytotoxicity index of the nanoparticle complexes were evaluated by MTT assay. Our proposed matrix for immunoglobulin adsorption is adaptable for applications towards plasma perfusion for removal of immunoglobulin.

2 Materials and methods

2.1 Synthesis of tryptophan/hydroxyapatite complexes

Disodium hydrogen phosphate (Na_2HPO_4), calcium chloride ($CaCl_2$), tri sodium citrate ($Na_3C_6H_5O_7$), tryptophan and β-cyclodextrin (β-CD) were purchased from Sigma Chemicals (Bangalore, India). All chemicals utilised were of reagent grade. In an earlier study we have shown the preparation of cyclodextrin complexed hydroxyapatite nanoparticles referred to as H in this manuscript (Victor and Sharma 2011). Briefly 0.1834 g of calcium chloride was dissolved in 100 ml of distilled water. Then 20 ml of fresh conjugate base prepared with 5.4 wt% tri sodium acetate was added and the reaction stirred for 5 min. To this solution 2.2% of 100 ml Na_2HPO_4 and 20% of 100 ml cyclodextrin solution was added dropwise from a burette and the stirring maintained for 24 h. The particles obtained were collected and the samples coded as H. The above optimized preparation technique was now modified by adding drop by drop varying concentrations of tryptophan water solutions (0, 100, 200 and 300 mg%) respectively and the stirring maintained for 24 h. The resulting complexes obtained were washed with distilled water, centrifuged and lyophilised. The complexed particles obtained were collected and the samples coded as HT1, HT2 and HT3 respectively. Thus H stands for the cyclodextrin complexed hydroxyapatite nanoparticle and HT1, HT2 and HT3 stands for the modified H complexes prepared by adding 100, 200 and 300 mg% of tryptophan to H, respectively.

2.2 Characterisation of the complexes

2.2.1 X-ray diffraction

The synthesized complexes were characterized by X-ray powder diffraction (XRD) method (Bruker AXS, X-ray diffractometer, reflection mode, Japan) using CuKα radiation with tube voltage 40 kV and 35 mA of tube current. Crystallographic identification of the H/Tryptophan complexes was accomplished by comparing the experimental XRD patterns to standards compiled by the Joint Committee on Powder Diffraction and Standards (JCPDS; HA, card #09-0432). The average crystalline size of the samples

was calculated from the XRD data using the Scherrer formula. The peak at 25.9° (002), was fit to define its full width at half maximum intensity ($B_{1/2}$(rad-2θ)):

$$D = 0.9\lambda / B_{1/2} \mathrm{Cos}\ \theta$$

where D is the crystal size, as calculated for the (hkl) reflection, λ is the wavelength of CuK_α radiation ($\lambda = 1.54$Å), and k is the broadening constant varying with crystal habit and chosen as 0.9 for the elongated apatite crystallites.

2.2.2 Transmission electron microscopy

The morphology of the complexes was determined by Transmission Electron Microscopy (TEM, Philips CM12 STEM, Netherlands). For TEM analysis, the powder sample was ultrasonically dispersed in ethanol to form dilute complexes and then a few drops were deposited on the carbon coated copper grids.

2.2.3 Dynamic light scattering

Dynamic light scattering (DLS) method (Malvern nanoseries zeta sizer) was utilized for the measurement of hydrodynamic size. The synthesised nanoparticle complexes were suspended in double ionised water and subjected to size measurement. All experiments were performed in triplicate.

2.2.4 Zeta potential

The surface charge of the prepared complexes was characterized by measurement of zeta potential. The zeta potential was measured by using the Zetasizer ZS (Malvern Instruments, Malvern, UK). Analysis time was about 1 min at 25°C and the average zeta potential (mV) was determined.

2.2.5 Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared spectra were recorded by diffused reflectance mode using a Thermo Nicolet, 5700, Germany spectrometer using KBr pellet technique. The FT-IR spectra were obtained in the region 400–4000 cm^{-1}. The functional groups present in the complexes were characterized and identified by their peaks as obtained in the spectra.

2.2.6 Differential scanning analysis

Differential scanning analysis (DSC) of the complexes was carried out using a DSC Thermal Analyser (SDT-2960, USA) with a temperature range of 100–500°. Samples were sealed in a platinum pan for analysis with an empty pan as reference. Thermograms were recorded at a scanning speed of 10°C/min under a nitrogen stream.

2.2.7 Thermal gravimetric analysis

The thermal behaviour of the prepared complexes was determined by a thermogravimetric analyzer (TA, USA) at a heating ramp rate of 10°C/min in nitrogen flow using platinum crucible.

2.3 Cytotoxicity studies—MTT assay

The Cell viability is a significant parameter to be evaluated in order to determine any toxic effect of the tryptophan/H complexes. In order to check the cytotoxicity of various concentrations of tryptophan complexed H complexes, MTT assay was done on L929 cell lines. These cells were seeded in culture flasks with DMEM medium along with 10% FBS and incubated for 24 h at 37°C in 5% CO_2 and 95% humidity to attain a cell growth of 80% confluency. Prior to the experiment, the cells were trypsinized and transferred to a 24-well cell culture plate at a density of 1×10^5 cells/well. The cells were exposed to a series of different concentrations of uncomplexed and complexed H particle (2.5–20 μM) for 48 h incubation followed by addition of 100 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution(0.5 mg/ml) and incubated for 3 h at 37°C. To dissolve the formazan crystals formed, 300 μl of DMSO was added per well and kept for 15 min at 37°C. The cells treated with the medium were used as positive control. The absorbance was measured at 620 nm using a plate reader (Finstruments Micro plate Reader USA).

$$\text{Cell viability} = [\mathrm{Abs}]_{\mathrm{sample}} / [\mathrm{Abs}]_{\mathrm{Control}} \times 100.$$

2.4 Electrophoresis analysis of proteins

Protein adsorption was studied by Polyacrylamide gel electrophoresis (SDS–PAGE). Plasma was obtained by the centrifugation of fresh blood (containing anticoagulant 3.8% sodium citrate) at 750 rpm. The samples H, HT1, HT2 and HT3 (1 g) were dispersed in 100 μl saline having a size of 100–300 nm and incubated with 200 μl plasma for an hour and then recovered by centrifugation. The proteins in the supernatant were then separated by subjecting to discontinuous native-PAGE method of Laemmli [20]. Electrophoresis was carried out at 100 V for 90 min using Mini-PROTEAN II electrophoresis cell (Bio-Rad, CA, USA). The gel was digitalized using an image analyzer (LAS 4000, Fuji) and the densitometry scans were done with the software Multi Gauge V3 and the peak heights were compared using the same soft ware.

2.5 Quantitative analysis of proteins

Human serum albumin, human fibrinogen and human γ-globulin were procured as lyophilised powders from Sigma (Bangalore, India) The buffer used in the protein adsorption experiments was phosphate buffered saline solution of pH 7.4 having a concentration of 0.01 M. All salts used in the buffer preparation were of reagent grade. The amounts of albumin, γ-globulin and fibrinogen in the isolated protein solutions before and after adsorption were estimated using Lowry's method [21]. Initially calibration curves were plotted for albumin, fibrinogen and γ-globulin from different known concentrations of protein solutions [19].

The quantification of γ-globulin protein on H was carried out by immersing 50 mg of particle in 10 ml of 25 mg % γ-globulin solution. At time intervals of 5, 15, 30 and 60 min, solutions are withdrawn and the amount of protein in the solution were obtained by Lowry method by measuring the absorbance values at the maximum observed at $\lambda = 750$ nm using UV spectrophotometer (Varian). The same procedure was applied for the HT1, HT2 and HT3 complexes. All experiments were carried out in triplicate. The concentration of the γ-globulin protein adsorbed was calculated by using the calibration curve obtained for pure γ-globulin.

Similar methods were adopted for studying the adsorption of albumin and fibrinogen by the HT1, HT2 and HT3 modified nanoparticle complexes. The quantification of albumin and fibrinogen protein was carried out by immersing 50 mg of the respective samples in 10 ml of 25 mg % albumin and 25 mg % fibrinogen solutions respectively. The concentration of the albumin and fibrinogen protein adsorbed was calculated by using the calibration curve obtained for pure albumin and fibrinogen, respectively. All experiments were carried out in triplicate.

2.6 Estimation of IgG adsorption

The amount of IgG adsorbed was calculated by quantitative turbidimetric assay using a standard IgG kit. The concentrations of IgG in the samples were measured from the calibration plot derived from known concentrations of IgG. The amount adsorbed was calculated by taking the difference between the pre and post adsorption protein values.

2.7 Statistical data analysis

Mean and standard deviation was evaluated from triplicate samples for protein adsorption and immunoglobulin estimation studies.

3 Results

The XRD patterns of H and Tryptophan/H complexes with varying concentrations of tryptophan are displayed in Fig. 1. All reflections observed for H including the characteristic peaks at $2\theta = 26.12°$ and 32.13° corresponded well to those expected from the hydroxyapatite structure (JCPDS 09-432) [22] and pure β-CD at 2θ values of 4.75°, 15° and 35.9° [19]. The presence of tryptophan in its crystalline form exhibits main diffraction peaks at 2θ values of 5, 9.9, 14.7, 18, 19, 34, and 45° respectively. The diffractograms of HT1, HT2 and HT3 show small peaks at 10.1°, 20° and 47° which are characteristic peaks of the tryptophan and correspond to peaks of pure tryptophan at 2θ values of 9.9°, 19° and 45°.

The bright field transmission electron microscopic images of the H nanoparticles and HT1 complex in as synthesized condition are shown in Fig. 2. The particles were thin and long with nano-plate like morphology. The sample preparation for analysis in TEM was done by drop cast method. We reason that the dark stripes visible in the image may be due to the transmitted electrons through multiple layers of the sample. Thus they are a shadow artefact of multiple nanoparticles in the electron beam. The Fig. 2c at higher resolution reveals a cloud of complexing protein. The protein layer appears like a grey shadow around the dark particles.

The size of the H and the Tryptophan/H complexes HT1, HT2 and HT3 were further studied by DLS analysis. Table 1 summarises the size and zeta potential of the complexes with increasing concentration of tryptophan. The average hydrodynamic diameter of the H particles was found to be 135 ± 2.3 nm. The size of the complexes were found to be larger than H and in the order HT < HT2 < HT3. The particle size of the H3 complex was found to be in the range

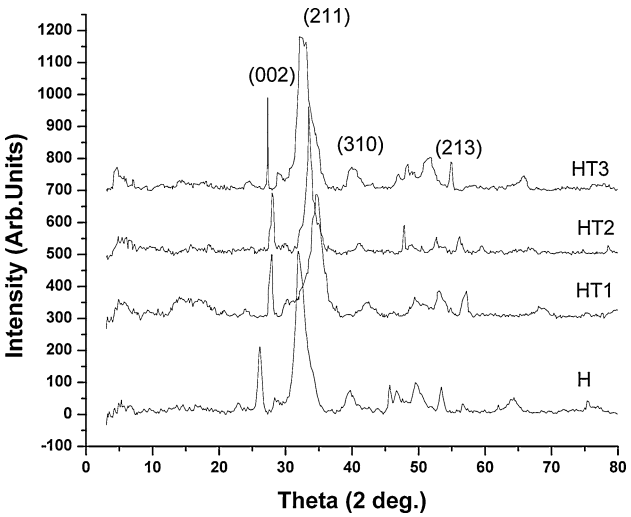

Fig. 1 XRD patterns of H, HT1, HT2 and HT3 complexes

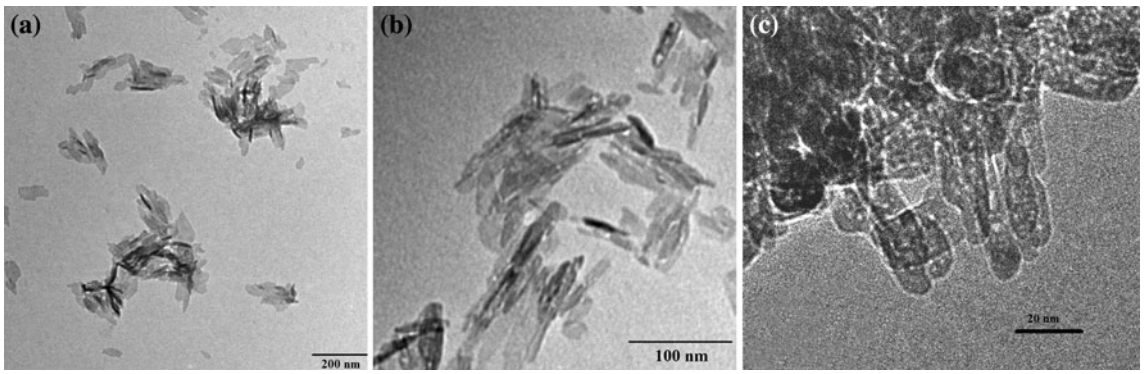

Fig. 2 TEM images of H nanoparticle (**a**) and HT1 complexes (**b, c**)

Table 1 Size and Zeta potential of H, HT1, HT2 and HT3 complexes

Sample	Size (*Z*-average) (nm)	PDI	Zeta potential (mV)
H	135	0.152	−30
HT1	238	0.167	−21.4
HT2	255	0.213	−14.7
HT3	278	0.149	−7.7

278 ± 7.9 nm. To elucidate the surface charge of the tryptophan/H complexes, zeta potential measurements were carried out. In the prepared complexes the values of zeta potential increased with increasing amounts of tryptophan as observed in Table 1.

FT-IR evaluation displayed in Fig. 3 indicates H and other tryptophan modified complexes with different functional groups stacked, respectively. The IR of H displayed peaks at 602, 900–1200, 3015, 3440 and 1624 cm^{-1} respectively. The spectrum of tryptophan shows characteristic stretching of carboxyl group at 1630 cm^{-1}, NH group at around 3400 cm^{-1} and CH_2 group at 2914 and 2850 cm^{-1} respectively [23]. The spectra of HT1, HT2 and HT3 show all the characteristic bands of H and additionally a distinct shoulder at 1590 cm^{-1}. The DSC thermograms of Tryptophan (indicated as T in Fig. 4), H and the complexes HT1, HT2 and HT3 are represented in Fig. 4. An endothermic peak was observed around

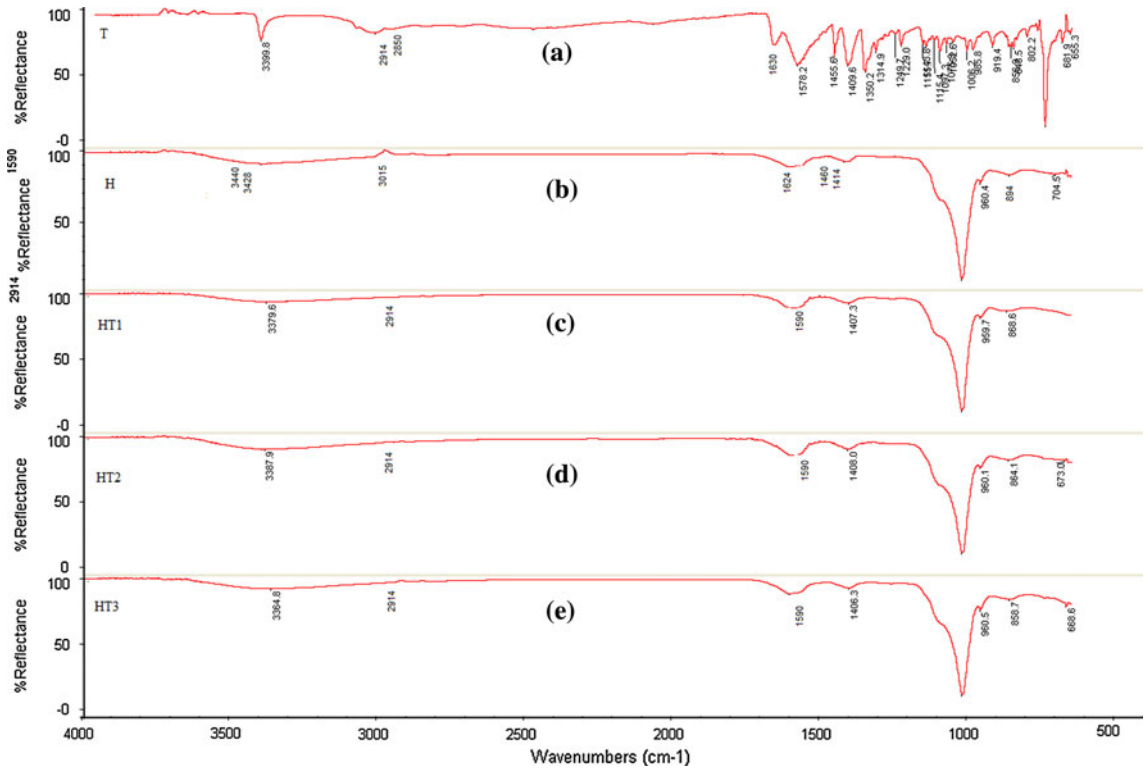

Fig. 3 FTIR spectra of Tryptophan (*a*), H (*b*), HT1 (*c*), HT2 (*d*) and HT3 (*e*) complexes

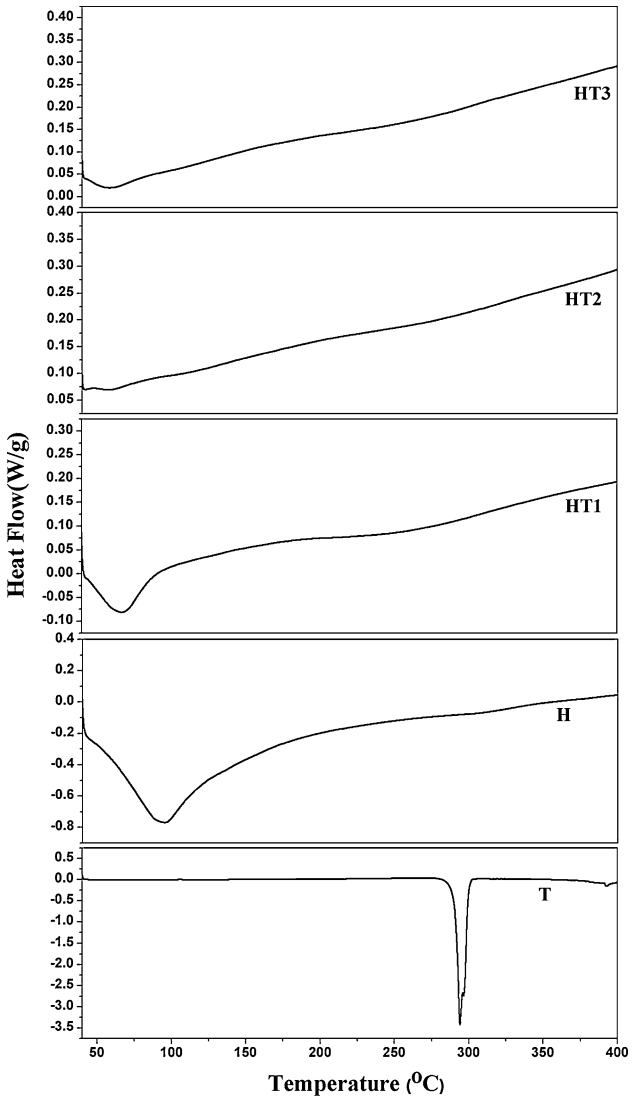

Fig. 4 DSC curves of T, H, HT1, HT2 and HT3 complexes

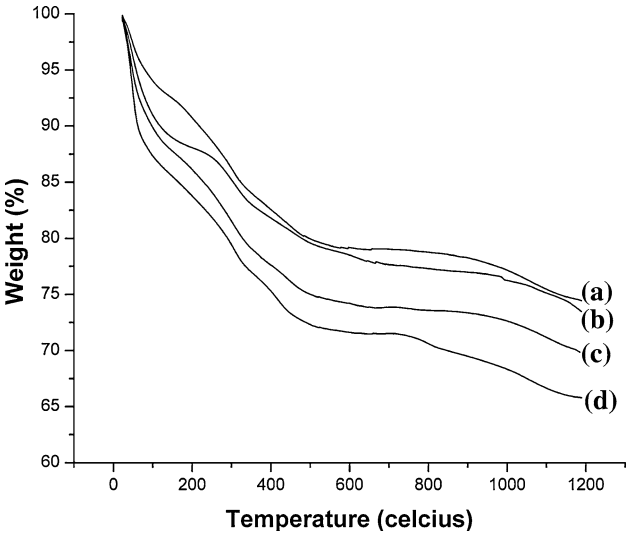

Fig. 5 TG Curves of H (*a*), HT1 (*b*), HT2 (*c*) and HT3 (*d*) complexes

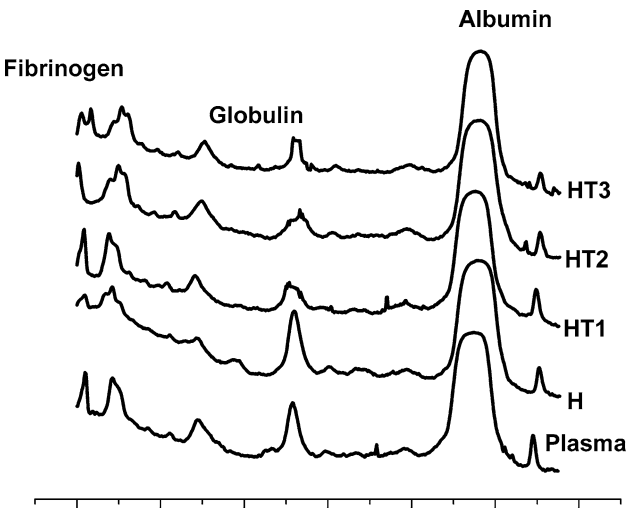

Fig. 6 Scanning photometry of the gel for plasma incubated with H and HT1, HT2 and HT3 complexes

100–130°C in H, HT1, HT2 and HT3 samples. The thermogram of pure tryptophan shows that thermal decomposition started at 270°C and melting occurred near 290°C respectively.

Figure 5a shows the TG curve of the H nanoparticle. The weight loss comprises of three stages. Figure 5(b, c and d) shows the TG curve of the tryptophan modified H sample HT1, HT2 and HT3, respectively. The weight loss associated with b, c and d (HT1, HT2 and HT3, respectively) was obviously higher than the weight loss of the sample 'a' (H nanoparticle) prepared without tryptophan. The thermal decomposition of tryptophan occurs in the range of 218–710°C through three distinct decomposition stages; at 243, 250 and between 270 and 440°C. This confirms the presence of tryptophan in the modified H nanoparticle complexes HT1, HT2 and HT3.

Protein adsorption by PAGE reveals a preferential and selective adsorption of γ-globulin from the plasma proteins as shown in the densitometry scan of the treated plasma (Fig. 6). It seemed that fibrinogen adsorption was negligible, and albumin adsorption from the plasma seemed to be similar, onto the modified particles.

The relative amounts of plasma protein adsorbed have been further substantiated by quantification using Lowry method. The adsorption isotherms for albumin, fibrinogen and γ-globulin and amounts of the various proteins adsorbed on native hydroxyapatite and cyclodextrin complexed hydroxyapatite were initially determined [19]. The amounts of the various proteins adsorbed onto the unmodified H and tryptophan complexed H particle elucidated from isolated

Table 2 Amount of proteins adsorbed onto H and HT1, HT2 and HT3 complexes from isolated protein solution

Particles	Albumin (mg/g)	Fibrinogen (mg/g)	γ-globulin (mg/g)
H	0.616 ± 0.03	0.232 ± 0.008	0.1404 ± 0.032
HT1	0.622 ± 0.026	0.250 ± 0.02	0.7522 ± 0.001
HT2	0.593 ± 0.008	0.28 ± 0.028	0.7047 ± 0.009
HT3	0.612 ± 0.025	0.267 ± 0.024	0.6719 ± 0.004

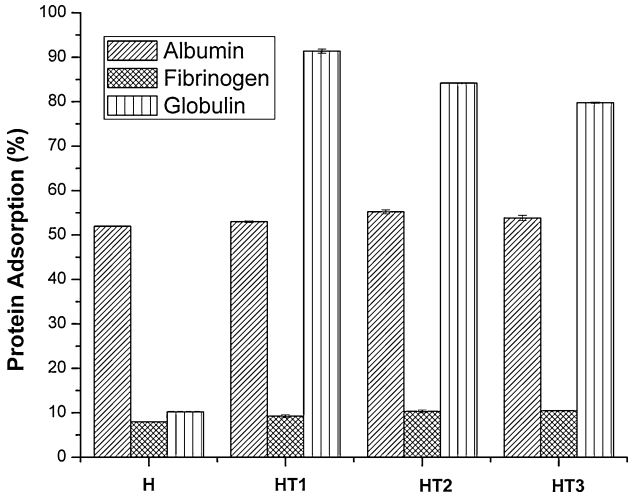

Fig. 7 Proteins adsorbed on H, HT1, HT2 and HT3 complexes (SD, $n = 3$)

protein solutions of equal concentrations (25 mg %) in 1 h is presented in Table 2.

The γ-globulin adsorption of tryptophan complexed H particle was significant compared to that of the unmodified particles. When compared to albumin and fibrinogen there was an appreciable increase in the adsorption of γ-globulin. However the adsorption of γ-globulin was found to decrease with an increase in concentration of tryptophan in the complexed H particles as shown. The adsorption of tryptophan was found to marginally decrease in the order HT1 > HT2 > HT3 as indicated in Table 2.

Figure 7 gives the % of protein adsorbed onto unmodified and tryptophan complexes from the isolated protein solutions. The figure clearly illustrates the similar observation of substantial γ-globulin adsorption when compared to albumin with tryptophan complexed H particles. It's seen that in both the modified and unmodified particles, the albumin adsorptions were comparable. The fibrinogen adsorption was fairly low for both modified and unmodified particles. For the treatment of certain autoimmune clinical cases the preferential adsorption of IgG for removal would be an advantage. Since Nano gels of isopropyl acrylamide have been used for protein loading and release studies,

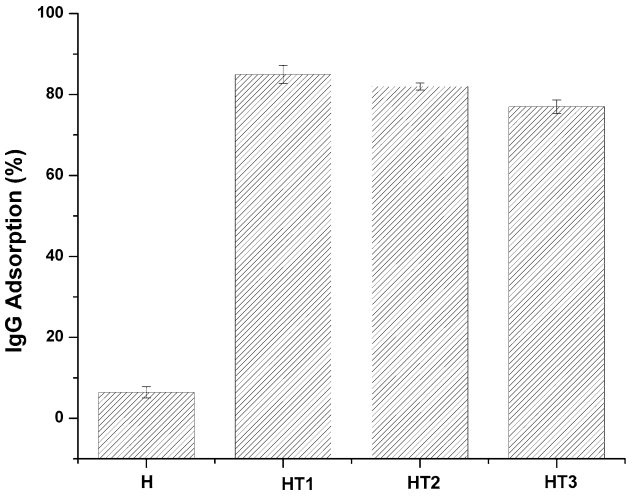

Fig. 8 Immunoglobulin adsorbed on H, HT1, HT2 and HT3 complexes (SD, $n = 3$)

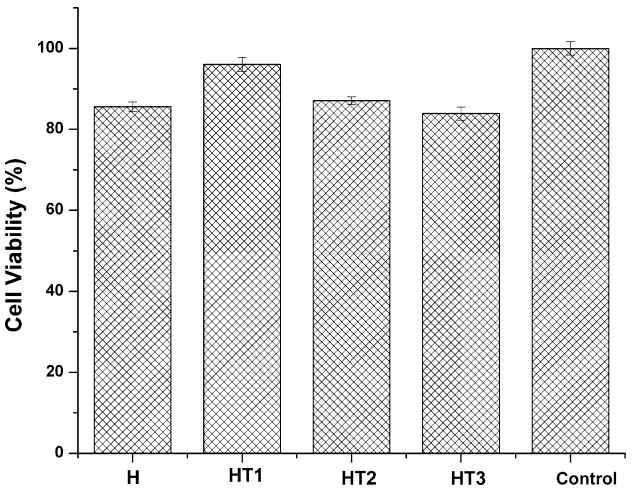

Fig. 9 Cytotoxicity of H, HT1, HT2 and HT3 against L 929 cells at polymer concentration of 100 µg/well; $n = 3$

tryptophan complexed nano particles could also be used for similar application [24].

The IgG adsorption capacities on unmodified H nanoparticles were compared with that of tryptophan complexed H particles HT1, HT2 and HT3 respectively. Figure 8 showed the % adsorption of IgG on unmodified and modified H particles. IgG adsorption was significantly increased in the case of tryptophan complexed particles compared with the uncomplexed particles. The in vitro cytotoxicity of the complexes was checked on L929 cell lines by MTT assay. All the tryptophan/H complexes were found to be non-toxic and showed >80% viability as indicated in the Fig. 9.

4 Discussion

The use of tryptophan to modify hydroxyapatite for use as a matrix for selective immunoglobulin adsorption has been described. The results obtained in this study were comparable to those present in literature. There is limited literature available on the use of hydroxyaptite nanoparticles for specific immunoglobulin adsorption. Hydroxyapatite microspheres immobilised with phenyalanine was found to selectively bind on hydrophoblic sites in the immunoglobulin molecule [17]. The selectivity in adsorption onto the modified microspheres could be enhanced by reducing the size of the microspheres [17]. This present study thus facilitated an enhancement in the adsorption of immunoglobulin by increasing the surface area available by promoting the use of nanoparticles. The XRD patterns of the prepared nanoparticle complexes depicted that the interaction between hydroxyapatite and tryptophan is of hydrogen bonding nature involving the –OH of apatite and the –COOH of tryptophan [25]. The average crystalline size of the H particles as estimated using Scherrer formula with (002) diffraction peak has been found to be 20 nm. The line broadening of the (002) peak was used to evaluate the crystalline size since the peak is well resolved and d_{002} values are related to crystal size in the broader aspect of hydroxyapatite crystallites. The average particle size of the prepared complexes was found to be directly proportional to the concentration of tryptophan. The presence of –COOH of tryptophan facilitates the formation of cross-links among themselves and also exhibits hydrogen bonding interactions with the –OH of hydroxyapatite and could be responsible for the increase in size of the complexes. So an increase in concentration of tryptophan will lead to more adsorption of ions leading to larger size as estimated by DLS measurements.

The measurement of zeta potential of the complexes allows predictions about the stability of prepared nanoparticles. Usually particle aggregation is less likely to occur for charged particle with optimum zeta potential due to electrostatic repulsions. The presence of tryptophan induces positive charges on the synthesised tryptophan/H complexes. Thus the prepared complexes HT1, HT2 and HT3 were found to be stable in nature. A similar observation of induction of positive charges was recently reported on the surface of gold nanoparticles [26].These positive charges impart stability to the gold nanoparticles. Since our prepared nanoparticle complexes have positive zetapotential, it can be inferred that the prepared complexes have adequate stability.

The FT-IR spectrum of H is identical to the representative spectrum of hydroxyapatite with phosphate stretching bands at about 900–1200 cm^{-1} and phosphate bending at 602 cm^{-1} [27]. The broad bands around 3015, 3440 and 1624 cm^{-1} are associated with the presence of water molecules in the sample. The characteristic peak of the hydroxyl stretching vibration at 3428 in the H sample is due to the coordination interaction between –OH of β-CD and the Ca^{2+} of the HA [19]. The spectrum of tryptophan shows characteristic stretching of carboxyl group at 1630 $cm^{-1,}$ NH group at around 3400 cm^{-1} and CH_2 group at 2914 and 2850 cm^{-1} respectively [28]. The band at 1630 cm^{-1} obscures the appearance of NH bending in the region 1590–1640 cm^{-1}. The spectra of HT1, HT2 and HT3 show all the characteristic bands of H thus signifying that the presence of tryptophan does not alter the spectra significantly. Additionally they also show the characteristic asymmetric CH_2 stretching of tryptophan at 2914 cm^{-1}. The distinct shoulder at 1590 cm^{-1} can be attributed to the involvement of carboxylate ion of tryptophan in hydrogen bonding with the hydroxyls on the surface of the hydroxyapatite molecules [25]. Hydrogen bonding is known to lower the stretching frequency and hence characteristic stretching of carboxyl group is shifted to 1590 cm^{-1}. Thus the characteristic band at 1590 cm^{-1} confirms the formation of hydrogen bonding between tryptophan and H in all the modified H complexes HT1, HT2 and HT3 respectively. The presence of the carbonate bands around 890, 1410 and 1460 cm^{-1} in all the spectra are due to the presence of carbonate ions in H and modified H complexes [28]. Carbonate containing hydroxyapatite has been shown to have better bioactivity due to it similarity with human bone. So the modified complexes prepared in the study are expected to demonstrate good compatibility.

The presence of an endothermic peak in the DSC around 100–130°C in H, HT1, HT2 and HT3 could be due to the presence of trace amounts of water in the samples. The modified H complexes containing tryptophan did not reveal any melting endotherm. This could be attributed to the hydrogen bonding that leads to complex formation between the H nanoparticle and the tryptophan. The TG data reveal that the weight loss comprises of three stages. The weight loss at first stage (below 100°C) is probably the evaporation of the absorbed water, the second stage is attributed to the loss of crystal water (200–400°C) and the decomposition of cyclodextrin present, and the third stage is due to the removal of constitutional water of hydroxyapatite. Figure 5(b, c, d) shows the TG curve of the tryptophan modified H sample HT1, HT2 and HT3 respectively. The thermal decomposition of tryptophan occurs in the range of 218–710°C through three distinct decomposition stages; at 243, 250 and between 270 and 440°C. This confirms the presence of tryptophan in the modified H nanoparticle complexes HT1, HT2 and HT3. This incremental weight loss observed in HT1, HT2 and HT3 is attributed to the decomposition of tryptophan present in the samples. Invitro cytotoxicity studies are an important factor in determining

the compatibility of particles for *invivo* applications. The MTT assays reveal that the complexes HT1 and HT2 showed enhanced viability when compared to H, and HT3 showed a marginal decrease in viability. More than 90% cells were viable in the HT1 complex. Thus it has been confirmed by the in vitro cytotoxicity studies that the tryptophan complexed H nanoparticles are non-toxic in nature.

Ceramic particles like hydroxyapatite, zirconia and alumina have been employed to study the adsorption behaviour of proteins from diluted human plasma with regard to total protein binding capacity, relative binding capacity for specific proteins and flow-through and desorption patterns [29]. Hydroxyapatite was found to have higher adsorption and binding capacity of plasma proteins compared to zirconia and alumina. However hydroxyapatite adsorbed 14% of albumin and 30% of immunoglobulin respectively. It has also been observed that immunoglobulin is adsorbed more onto hydroxyapatite compared to other immunoproteins [30]. In the present study the introduction of tryptophan to form hydroxyapatite tryptophan complexes resulted in over 70% immunoglobulin adsorption as observed in the protein adsorption studies.

Protein adsorption to any substrate from a mixture of protein could be directly correlated to that from plasma [31]. Studies from a mixture of albumin, γ-globulin and fibrinogen show an increased γ-globulin adsorption for the complexes HT1, HT2 and HT3. The protein bands were identified with plasma standards. The difference in the peak intensity of plasma before and after adsorption clearly demonstrated the enhanced protein adsorption. It seemed that fibrinogen adsorption was negligible, and albumin adsorption from the plasma seemed to be similar, onto the modified particles. On comparison with the peaks of γ-globulin in plasma and the sample after adsorption with modified particles, the γ-globulin adsorbed onto the modified particles were more than on the unmodified particles. The PAGE results thus indicate that a fairly reasonable adsorption occurred in the case of tryptophan modified particles. This specific affinity based adsorption has been found to depend on both the hydrophobic force of the side chain and the anionic charge of the carboxylic acid in tryptophan. This affinity of immunoglobulin towards tryptophan has previously been reported in a similar study carried out from patients with Fischer syndrome [32]. The tryptophan immobilised PVA gel adsorbed greater amounts of immunoglobulin than the unmodified one. Several patients with Fisher's syndrome therefore were given immunoadsorbent therapy using the IM-TR column without adverse reactions.

As mentioned earlier the adsorption of γ-globulin was found to decrease with an increase in concentration of tryptophan in the complexed H particles as shown. This decrease in adsorption could be explained as follows. At higher concentrations of tryptophan the molecules get adsorbed on to hydroxyapatite through their carboxylic terminals adopting a vertical orientation [25]. So less number of carboxyl groups is available for tryptophan for affinity based interaction with globulin. This decrease in the anionic charge of the carboxylic acid could explain the marginal observed reduction in the adsorption of globulin at higher concentrations of tryptophan. In general, several factors influenced the particle-protein interaction like the number of electron- donating groups on the protein surface, the hydrophobicity, the protein concentration and the type of metal ions [33]. Here the specific immunoglobulin adsorption has been found to depend on both the hydrophobicity and the anionic charge of the carboxylic acid in tryptophan. This attribute favoured a closer interaction between the protein and the tryptophan complexed H particle thus enhancing immunoglobulin adsorption. However a marginal decrease in adsorption of IgG has been observed due to the observed orientation change of tryptophan at higher concentrations as described previously. To summarise; from the protein adsorption experiments it is evident that the affinity of the HT1 complex for immunoglobulin protein is greater than that of the unmodified H particle. Tryptophan the amino acid complexed with H nanoparticle is highly hydrophobic and selectively binds to hydrophobic sites on the immunoglobulin molecule via physiochemical interaction based on a hydrophobic bond. Thus the synthesized complexed particles show the desirable immunoglobulin adsorption capability. The immunoglobulin and protein adsorption reported in this study with hydroxyapatite were comparable with results available in literature [34, 35] with other polymers. Adsorbents with immunoaffinity property are commonly prepared from particulate forms modified appropriately for a specific application. These devices are used successfully for the removal of pathogenic molecules from plasma or for the purification of proteins. So far different types of affinity supports have been reported in literature for human-IgG and protein adsorption which include protein A-immobilized PHEMA adsorbents, amino acid containing membranes copper immobilized cellulose membranes and imino diacetic acid functionalized adsorbents [36–38]. The literature available on the use of modified hydroxyapatite as a matrix for immunoglobulin adsorption is limited. The present study encompasses the advantages of higher surface area, compatibility, higher adsorption and binding capacity of hydroxyapatite along with the specific affinity of tryptophan for immunoglobulin to provide a suitable nanocomplex that can facilitate as a matrix for immunoglobulin adsorption. This alternative cost effective matrix may prove to be useful for removing immunoglobulins in plasma perfusion applications.

5 Conclusion

In the current study different levels of tryptophan have been complexed with cyclodextrin containing hydroxyapatite nanoparticles of size 20–30 nm. The size of the complexes as analyzed using DLS was between 150 and 280 nm and was thin and long with nano-plate like morphology. The protein adsorption experiments and the estimation of IgG by quantitative turbidimetric assay demonstrate the affinity of the prepared tryptophan complexes for immunoglobulin protein. Tryptophan the amino acid complexed with H nanoparticle is highly hydrophobic and selectively binds to hydrophobic sites on the immunoglobulin molecule via physiochemical interaction based on a hydrophobic bond. It appears that presence of cyclodextrin has a synergic effect in the adsorption of immunoglobulin proteins having affinity with tryptophan complexed hydroxyapatite.

Adsorbents with immunoaffinity property are prepared from particulate forms modified appropriately for a specific application. Since nanoparticles provide increased surface area available for attachment of ligands they can also be preferentially utilized for specific adsorption. The present study thus focuses on the use of modified nanosized hydroxyapatite as a matrix for affinity based immunoglobulin adsorption. The simplicity and low cost of the matrix may prove to be useful for removing immunoglobulin in plasma perfusion applications.

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