A PRACTICAL METHODOLOGY FOR IgG PURIFICATION VIA CHITOSAN BASED MAGNETIC NANOPARTICLES

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The objective of this investigation is to develop a simple, rapid, and practical separation system for IgG separation using magnetic chitosan nanoparticles. Magnetic nanoparticles were prepared by co-precipitation method and coated with a biocompatible polymer chitosan by varying molecular weights. Morphological analyses of magnetic chitosan nanoparticles were investigated by Scanning Electron Microscope (SEM) and Zeta-Sizer. Chemical structure of the nanoparticles was analyzed by Fourier Transform Infra-Red Spectroscopy (FTIR) and magnetic properties were investigated using Electron Spin Resonance (ESR) and Vibrating Sample Magnetometer (VSM). For the purification of IgG, a well-known protein Concanavalin A (Con-A), having a strong affinity to the IgG molecules, was immobilized onto magnetic chitosan nanoparticles. IgG purification was performed using a batch separation system made locally. It was observed that IgG can easily be purified from fresh human plasma with high efficacy, using a simple and inexpensive system prepared locally. Nanoparticle size, IgG concentrations and the Con-A concentrations were investigated to get an optimum separation efficiency.

1. Introduction

There is a significant research interest in magnetic nanoparticles, as they offer a large spectrum of applications arising due to their thermal, chemical, electrical, and magnetic field properties (Gu H.W. et al. 2006, Wang et al. 2007, Wu et al. 2006). All these properties are unique that contrast them from traditional nanoparticles and bulk materials. Further to these properties, they also show reduced toxicity and non-existent immunogenicity. Furthermore, it is easy to separate such nanoparticles from the reaction system (Cui et al. 2006). Magnetic nanoparticles are widely believed to have applications in biomedical field such as hyperthermia, cancer diagnosis cell therapies, magnetic resonance imaging, enzyme immobilization and cell separation studies (Föster et al. 2002; Zhang et al. 2002; Mangeney et al. 2002; Chang et al. 2005, Chen et al. 2006; Lu et al. 2007, Odabas et al. 2008). Furthermore, the use of magnetic separation systems in biological field offers an alternative against non-magnetic techniques such as gravitational or

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centrifugal separations (Safarik et al. 1999, 2009). There are many additional advantages of using magnetically separable particles as supports for biological molecules, such as enzymes, antibodies (Narasimhan, B. et al. 2011) and other bio-affinity adsorbents. (Li et al. 2002; Lowet al. 2007). Magnetic nanoparticles have multiple applications in the field of separation and purification technology (Yavuz et al., 2009, Safarik et al., 2004). One of the most important characteristics of these nanoparticles is to have a large volume of surface area (the smaller the size of the particle the larger the surface area) which leads to high binding capacity for the ligands. (Dios et al, 2010). Notwithstanding numerous advantages, there are some limitations in using magnetic nanoparticles, such as large ratio of surface area to volume leading to aggregation; a mechanism to reduce their surface energy due to strong magnetic attractions between particles (Chen et al., 2000; Maria et al., 2004). To prevent such aggregation, stabilizers such as surfactant, metal chelating agents, polymeric compounds are generally used (Yang et al., 2006). Various types of natural and synthetic polymers (i.e. polystyrene, polyacrylamide, polyvinyl alcohol, poly-lactic acid, alginate etc.) have been used in preparation of magnetic nanoparticles, due to presence of variety of surface functional groups which can be tailored to specific applications (Martin et al. 2004; Oster et al. 2001; Tong et al. 2001). Chitosan, poly (1-4)-2-amino-2-deoxy-D-glucan, is a well-known heteropolysaccharide (polycationic) with many important biological and chemical properties. It is biocompatible/non-toxic, hydrophilic, biodegradable, non-immunogenic and has remarkable affinity for many biomacromolecules (Crompton et al., 2007). Chitosan has been applied to many fields such as metal adsorption, enzyme immobilization, protein adsorption, controlled release of drugs for defense applications, etc. (Ngah et al. 2005; Mao et al. 2004; Chassary et al. 2004, Kavaz et al. 2011). Chitosan can further be used to provide highly biocompatible magnetic nanoparticles. The objective of this investigation is to prepare well-dispersed suspension of super-paramagnetic, Fe_3O_4, chitosan coated magnetic nanoparticles by co-precipitation method using a simple and inexpensive batch technique of IgG separation. The reported methodology has potential to be adopted by other laboratories and perhaps transition to large scale commercial unit.

2. Materials and methods

2.1 Materials

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, 99%), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$, 99%), and ammonia (NH_3) (Riedel, Germany) were purchased from Sigma (Taufkirchen, Germany). Ammonia was used as precipitator of ferric salts. Chitosan with different molecular weights (viz. 150, 450 and 650 kDa) were obtained from Fluka (Switzerland). Aqueous acetic acid (Carlo Erba, Italy) solutions were used as solvent for the chitosan. Sodium triphosphate pentabasic (Sigma Aldrich, ABD) was used as cross-linker. Water soluble carbodimide (WSC) [1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide], Phosphate Buffer Saline, MES buffer [2-(N-morpholino) ethane sulfonic acid and $C_6H_{13}NO_4S$] and glycine (pH 8) were purchased from Fluka (Switzerland). Concanavalin-A was used as model ligand for the nanoparticles (Sigma Aldrich, ABD). All other chemicals were reagent grade and used without further purification.

2.2 Preparation of Magnetic Chitosan Nanoparticles

Magnetic chitosan nanoparticles were prepared by using co-precipitation technique as reported elsewhere [Kavaz et al., 2010]. For this investigation, an optimized formulation, e.g., by nanoparticles size-size distribution and magnetic quality was used. In a typical procedure, magnetic chitosan nanoparticles were prepared as follows. First, chitosan was dissolved in 5 % acetic acid to obtain 0.5 % chitosan solution. The pH of the solution was adjusted to 4.6 - 4.8 by using 10 M NaOH solution. Then,1:2 M Fe^{2+}/Fe^{3+} iron salts were dissolved in tripolyphospahe (TPP) solution (Chitosan/TPP = 1:5). Solution of 3M ammonia was added to iron salts – TPP solution while the solution was stirring vigorously. The resulting solution was stirred for 1hr. at room temperature to ensure that the particles were entirely coated. The colloidal solution of magnetic chitosan nanoparticles was then centrifuged for 10 min at a speed of 9000 rpm. The

magnetic and non-magnetic particles were separated by using a neodium earth magnet. Later on, magnetic nanoparticles were washed with distilled water several times.

3. Characterization of magnetic chitosan nanoparticles

3.1 Morphological and Physicochemical Evaluations

The morphology of magnetic chitosan nanoparticles were carried out by using a scanning electron microscope (SEM, Jeol, Japan). The average particle size-size distributions and surface charges of the nanoparticles were determined by using a Zeta-Sizer (Malvern Instruments, Model 3000 HSA, France). Chemical structure of unadorned Fe_3O_4 nanoparticles and Fe_3O_4 loaded chitosan nanoparticles were analyzed using Fourier transform infrared spectroscopy (FTIR, Schimadzu, DR8101, Japan).

3.1.1 Magnetic Properties

Magnetic properties of unadorned magnetic nanoparticles and magnetic chitosan nanoparticles were evaluated with Electron Spin Resonance Spectroscopy (ESR) and vibrating sample magnetometer (VSM, LDJ 9600). The ESR measurements were performed at room temperature using spectrometer operating conditions at 9.3 GHz, 3,300 field set, 100 Hz field modulation, 1G peak-to-peak amplitude modulation and 1 mW microwave power to determine signal intensities, line widths, and g-values. All ESR signal intensities were performed using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) standard sample. The data were used to perform ESR spectrum to measure intensity versus magnetic field. In VSM measurements, an increasing magnetic field between 0–15000 Oe was applied over the nanoparticles for the determination of the degree of magnetism and the results were evaluated to calculate the magnetic quality of the nanoparticles in electron mass units (emu).

3.2 Ligand Immobilization onto the Magnetic Chitosan Nanoparticles

Magnetic chitosan nanoparticles were modified by using a model ligand having an affinity to the IgG molecules (i.e.Con-A). For the immobilization of Con-A to magnetic chitosan nanoparticles, a relatively well-known and widely used procedure was used. Briefly, 500 µg/ml Con-A was dissolved in 0.1M MES buffer (pH 5.2), mixed with functionally activated 50 mg/ml magnetic chitosan nanoparticles, and incubated in a rotator at 25°C for 24 hrs. After this immobilization step, particles were removed from the medium, washed several times with PBS (0.1 M K_2HPO_4–KH_2PO_4 with pH 7.4) and kept in PBS until use. Coupling efficiencies were determined by UV-spectrophotometer (Shimadzu, Japan). The release of Con-A from nanoparticles was evaluated by treating the nanoparticles with PBS for 24 hrs at room temperature. The released amount of Con–A was measured spectrophotometrically at 280 nm.

3.3 Adsorption of IgG from Aqueous Solutions

The batch separation was performed by using a very simple system prepared locally, which consists of a glass cylindrical tube over a permanent Neodymium earth magnet. Briefly, the tubes containing IgG suspension were allowed to interact with the magnetic chitosan nanoparticles for 10 minutes. The supernatant was then discarded and the precipitated part was collected after washing several times with PBS buffer.

3.4 Adsorption of IgG from Human Plasma

The adsorption of IgG from human plasma was studied batch-wise. Fresh human plasma (500 mL supplied from University Hospital, Hacettepe, Ankara) was used for all experiments and PBS (pH 7.4, 0.9% NaCl) was used for the dilutions. The Con-A immobilized-magnetic chitosan nanoparticles were incubated at 20°C for 10 min with 2 mL of human plasma. The amount of IgG

adsorbed through Con-A on the magnetic chitosan nanoparticles was determined by a solid-phase enzyme- linked immunosorbent assay (ELISA).The same process was also applied to the albumin and fibrinogen to determine the Con-A specificity. Total protein concentration was measured by using a total protein reagent.

3.5 Desorption and Reusability

The desorption of IgG was studied in a 2 M NaCl aqueous solution. First, IgG was allowed to interact with the nanoparticles by using abovementioned procedure (Con-A loading ratio was 70 mg/g and IgG concentration was 1 mg/ml). Then, magnetic chitosan nanoparticles were placed in the desorption medium and stirred with a rotator for an hour at room temperature. The total volume of the desorption medium was 50 mL. The final IgG concentration in the desorption medium was measured by UV-spectrophotometer at 280 nm. The desorption ratio was then calculated. Reusability of magnetic chitosan nanoparticles was determined by repeating the adsorption desorption cycle 5 times with the same nanoparticles.

4. Results and discussion

4.1 Characterization of Magnetic Chitosan Nanoparticles

4.1.1 Morphological and Physicochemical Evaluations

Microscope and Atomic Force Microscope. As shown in Figures 1 and 2 (a, b), magnetic chitosan nanoparticles are sphere like shape in geometry and their sizes are under 200 nm average values.
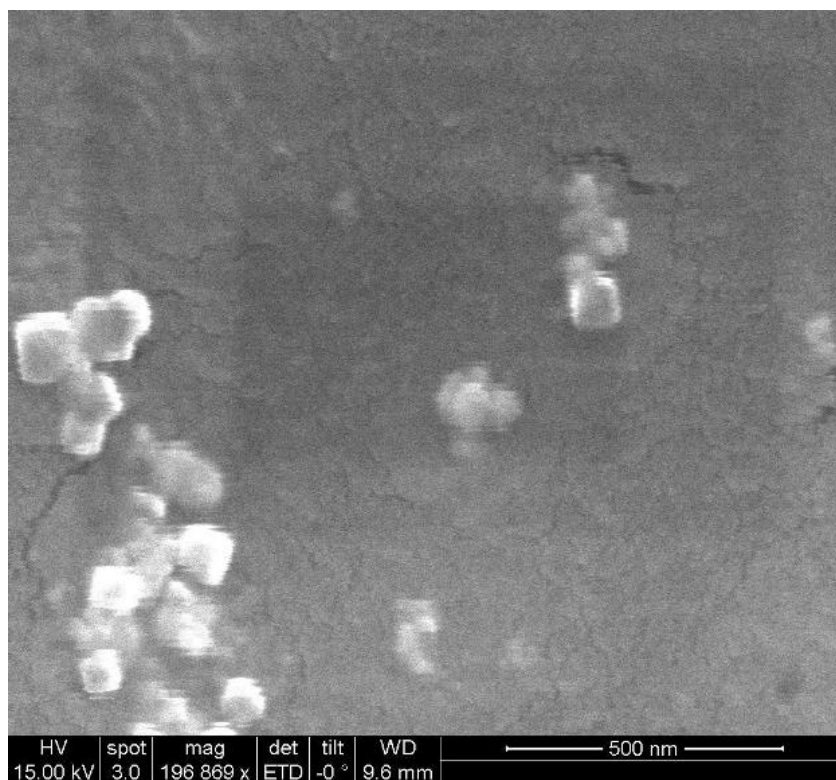

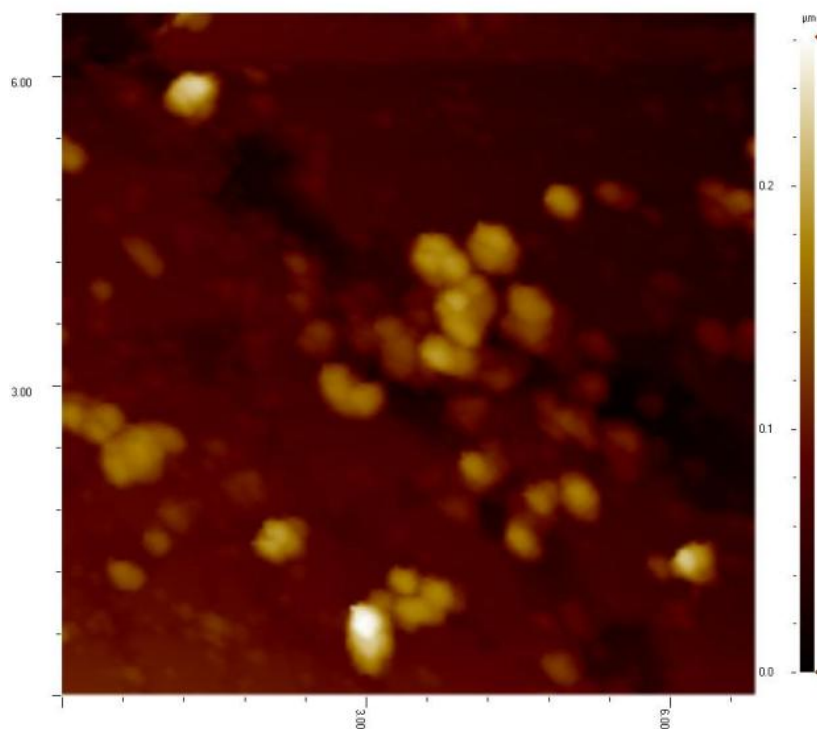*Fig. 1. SEM micrograph of magnetic chitosan nanoparticles*

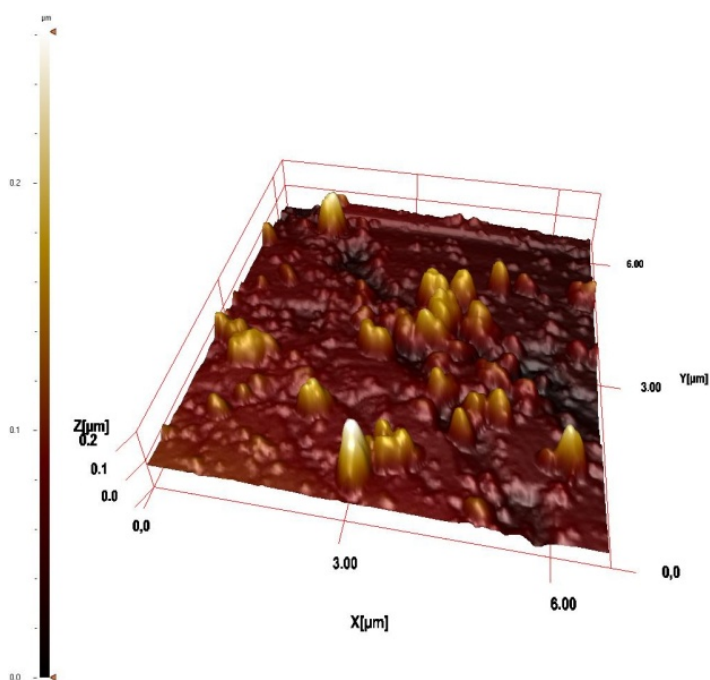*Fig. 2a. AFM image of magnetic chitosan nanoparticles*

Fig. 2b. 3D AFM image of magnetic chitosan nanoparticles

In additional to the morphological evaluations, size and distributions of magnetic chitosan nanoparticles were evaluated using a Zeta-Sizer. Some critical parameters, such as chitosan molecular weight (i.e.150, 450 and 650 kDa) and chitosan/TPP weight ratio (i.e., 0.5, 1.0 and 1.5) were also investigated to have optimum size and distribution values. All other parameters were kept constant during the evaluation. Stirring rate and ammonia concentartion also fixed at 2000 rpm and 3M respectively. Experimental data, as obtained, is summarized in Table 1.

Table 1:Effect of parameters on size-size distribution of magnetic chitosan nanoparticles.

Sample No	Chitosan MW	*chitosan/TPP*	Stirring Rate (rpm) and Ammonia Conc.(M)	Nanoparticle size (nm)
Effect of chitosan molecular weight on nanoparticle size				
1	LMW	*1.0*	2000 rpm/3 M	156.67±5.06
2	MMW	*1.0*	2000 rpm/3 M	260.33±0.33
3	HMW	*1.0*	2000 rpm/3 M	309.67±1.52
Effect of chitosan/TPP weight ratio on nanoparticle size				
4	LMW	*0.5*	2000 rpm/3 M	156.67±5.06
5	LMW	*1.0*	2000 rpm/3 M	371.33±20.13
6	LMW	*1.5*	2000 rpm/3 M	430.00±8.19

As seen in Table 1, the size of the magnetic chitosan nanoparticles increases with the increase of chitosan/TPP ratio. The observed behavior can be explained by a higher molecular weight of the chitosan polymers and by forming a large size due to crosslinking mechanism due to co-precipitation method.

4.2 Chemical Structure

Chemical structure of the chitosan polymer and magnetic chitosan nanoparticles were analyzed using FTIR. It was observed that OH groups were present both in the chitosan polymer and magnetic chitosan nanoparticles. Furthermore, at 1700 cm^{-1} both chitosan polymer and magnetic chitosan nanoparticles showed carbonyl band peak with similar intensities.

4.3 Magnetic Properties

ESR results of magnetic chitosan nanoparticles show that magnetic field intensity of the particles was found to be in the range of 3975 Gauss. The magnetic properties of magnetic chitosan nanoparticles were also analyzed using a VSM system. VSM results of magnetic chitosan nanoparticles show that both the magnetite and the magnetic chitosan nanoparticles were super-paramagnetic and no remanence was observed upon removal of magnetic field. (Kavaz et al, 2010)

4.4 Concanavalin-A Immobilization

Concanavalin-A (Con-A) was used for adsorption of IgG due to its specific interactions via carbohydrate residues of proteins. Con-A was covalently immobilized onto the magnetic chitosan nanoparticles through a coupling reaction between free amine groups of Con-A and the carbonyl groups. The Con-A immobilization efficiency of the nanoparticles was determined by UV spectrophotometer and was found ≈ 82 %.

4.5 Adsorption of IgG

To determine the adsorption of IgG, using an aforementioned procedure to remove IgG was used by (Con-A)-magnetic chitosan nanoparticles. During this experimental method, parameters such as size of nanoparticle, concentration of (Con-A) and initial IgG were selected to influence the IgG adsorption performance from the magnetic chitosan nanoparticles. Results and discussions are provided in the following subsections.

4.5.1 Effects of Nanoparticle Size

The size of magnetic chitosan nanoparticles were selected as one of the main parameter for IgG separation due to adsorption characteristics of the separation process. Due to smaller nanoparticles (<200 nm), they have prominently larger surface area, hence they have much higher binding capacities for the IgG molecules. In contrast, larger size nanoparticles (>200 nm) may aggregate and block specific binding regions of the nanoparticles resulting in a substantially reduced separation yield. Hence, such applications require agitation for larger nanoparticles to prevent aggregation. In this part of the study; average size of the magnetic chitosan nanoparticles were varied from 150 to 350 nm to investigate the effects of nanoparticles on IgG removal. The percentage of adsorbed IgG values is provided in Figure 3. It was observed that adsorbed IgG values were lower in relatively smaller and larger nanoparticles, while the highest adsorption was obtained for intermediate sizes (i.e., 200 nm). This observation may be postulated by presence of physical barriers during binding of IgG molecules on the magnetic chitosan nanoparticles via the ligand (i.e., Con-A) molecules.
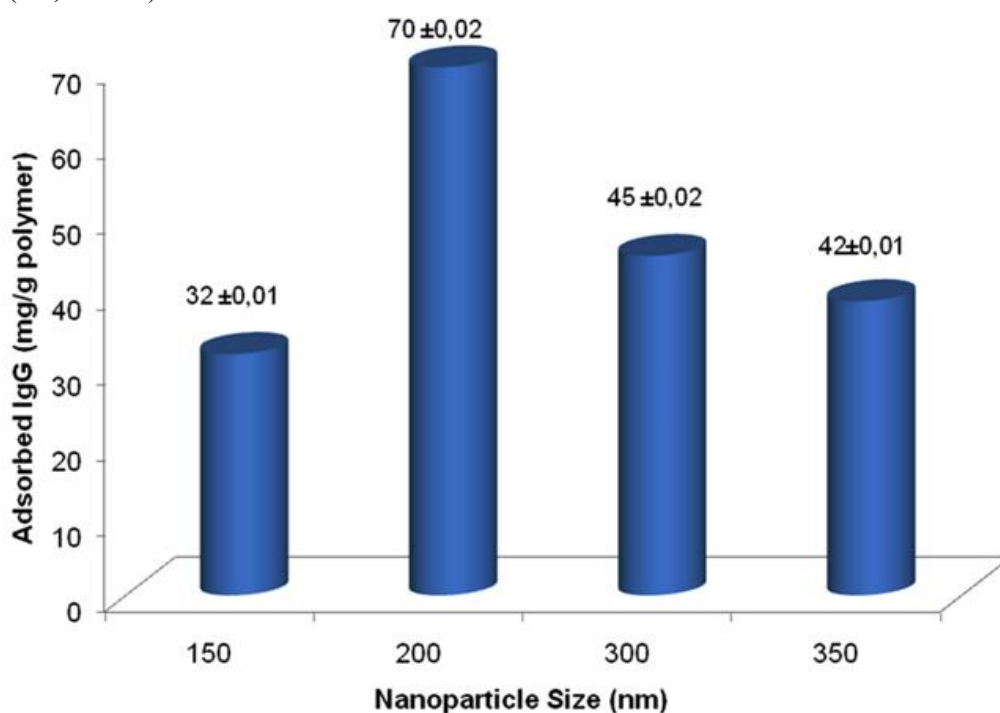

Fig. 3. Adsorption of IgG by changing nanoparticle sizes.

4.5.2 Effects of Con–A Concentration

As in the protein separation and purification techniques, the amount of recognizing molecule or ligand is one of the most important parameter. The amount of ligand molecules directly determine the capacity of the targeted molecules (i.e., the protein molecules to be purified). Therefore the Con-A protein concentration during the attachment of the Con-A onto the magnetic chitosan nanoparticles was investigated as another effective parameter for the IgG purification. For this purpose, first Con-A was attached onto the magnetic chitosan nanoparticles as described in the previous sections, using different initial Con-A concentrations (i.e. 150, 250, 400, 600 mg/ml). Con-A immobilized-magnetic chitosan nanoparticles were incubated with IgG solution (50 mg/ml) for 10 min. After this incubation period, purified IgG concentrations were analyzed by spectrophotometric measurements. Adsorbed IgG values were given in Figures 4. In this experiment, the adsorbed IgG amount was increased by an increase of Con-A concentration to a certain value (i.e., 400 mg/ml Con-A) as avidity interactions. Afterwards, even if the Con-A concentration increased, adsorbed IgG value remained constant and adsorbed IgG capacity was limited due to lack of possible interactions points on magnetic chitosan nanoparticles (Bayramoğlu et al., 2007). Maximum adsorbed IgG value obtained was ≈ 67 %, under the given conditions. At a point of 400 mg/ml Con-A value.
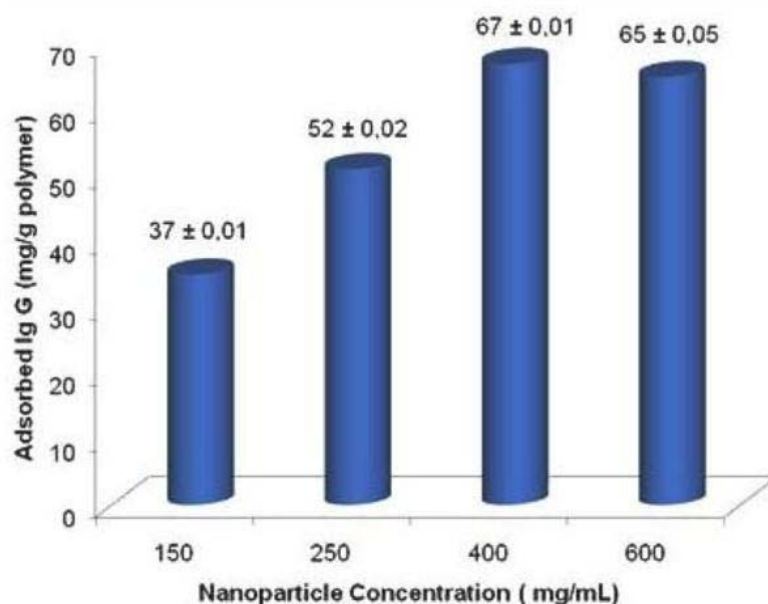

Fig. 4. IgG purification by changing Con-A concentration

4.5.3 Effects of Initial IgG concentration

Con-A has strong affinity to IgG molecules. Therefore initial IgG concentration is an important parameter on the adsorption of IgG. Initial IgG concentration was changed between 0 to 2 mg/ml IgG. The data obtained during the experimentation is provided in Figure 5. Adsorbed IgG ratio was increased initially and after the 1 mg/ml of initial IgG concentration the purification value was reached a maximum value. After this maximum value, adsorbed IgG ratio was decreased by increasing the IgG concentration. (Dawes C.C. et al, 2005)
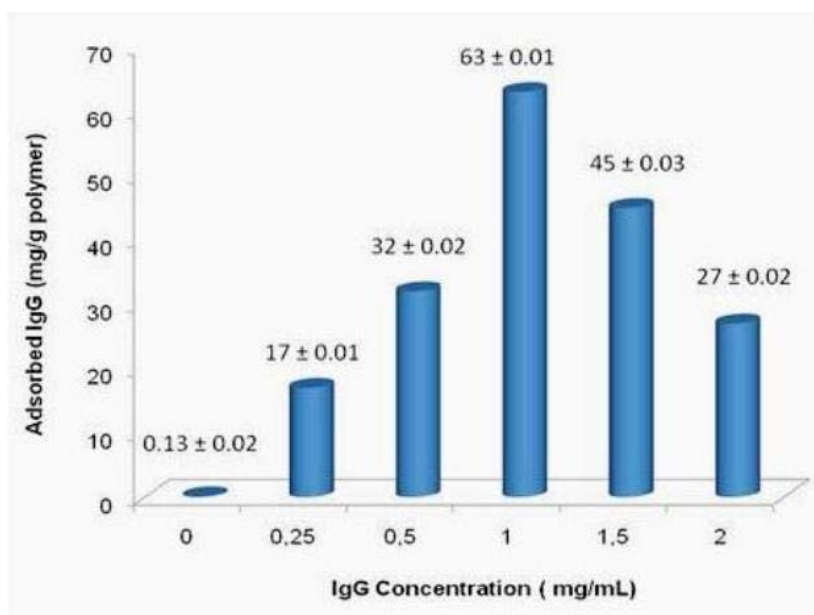
Fig. 5. Effect of IgG concentration on adsorption capacity

4.5.4 Effects of Ph

IgG Adsorption onto magnetic chitosan nanoparticles can affected by buffer systems. (Bowman and Leong, 2006) To clarify this point, adsorption studies were also carried out using different buffer systems like; MES (5.5-6.5), acetate (5.0-6.0) and phosphate (6.0-8.0). Figure 6 shows IgG adsorption capacity in these buffer systems at different pH values. It is clearly seen that the adsorption capacities vary between acidic and basic pHs. The maximum adsorption of IgG from the aqueous solution was in phosphate buffer, pH at 7.0 which is nearly close to common body reaction pH 7.4.
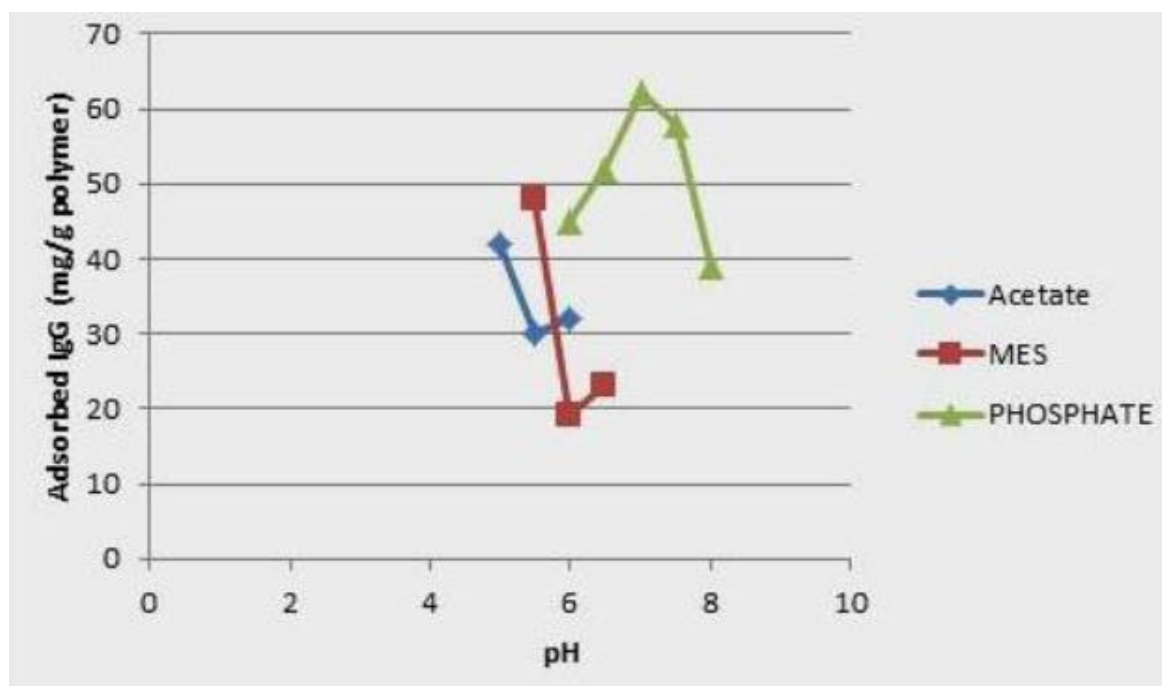

Fig. 6. Effect of pH on adsorption capacity

4.5.5 Effects of Ionic Strength

Ionic strength is a parameter, depended on the concentration of ions in a solution. So it can change with the change in the molar concentration of the solution. These phenomena can affect the stabilization of the nanoparticle in the solution and also adsorption of IgG from the solution. Figure 7 shows the effects of ionic strength to the adsorption capacity. The adsorption capacity was decreased by the increase of the NaCl in the solution. In fact, repulsive electrostatic forces form in a protein solution that prevent aggregation of the protein in the solution, thus helping the solubility of the protein. When the concentration of the salt in the solution increases it may decrease the protein solubility which also leads to less protein adsorption. In addition, higher ionic strength media display lower magnetic saturation which also leads to low separation yields. (Qui Xing-Ping, 2000, Laurent et al., 2008)
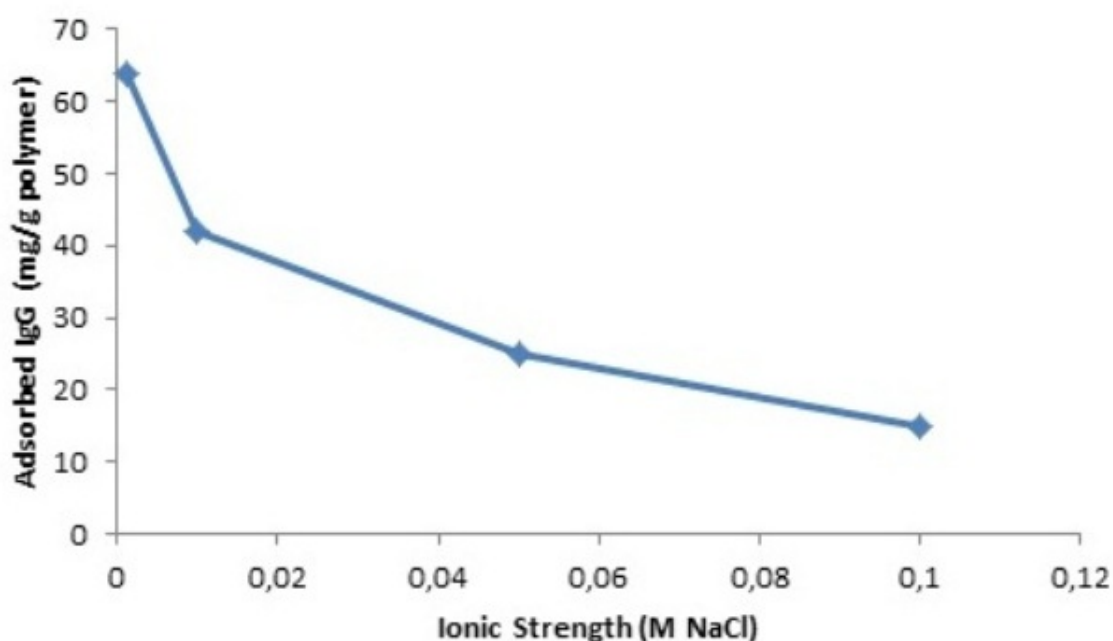

Fig. 7. Effect of ionic strength on adsorption capacity

4.6 IgG Adsorption from Human Plasma

The adsorption of IgG in human plasma was performed batch wise. Figure 6 shows the adsorption of IgG from human plasma which was performed with ELISA. There was a very low non-specific adsorption of IgG which was ≈ 0.7 mg/g polymer, whereas higher adsorption values were obtained as 69 mg/g polymer with 80.2 mg/g Con-A loading. The adsorption of IgG onto the magnetic chitosan nanoparticles was higher than these values obtained in studies in which aqueous solutions were used.

To obtain the Con-A specification, adsorption of other plasma proteins was also studied. The protein adsorption capacities were 72.0 mg/g for IgG, 2.3 mg/g for fibrinogen, and 3.4 mg/g for albumin. The total protein adsorption was determined to be 77.7 mg/g. The adsorption of other plasma proteins (i.e., fibrinogen and albumin) on the magnetic chitosan nanoparticles was negligible. Note that; when human plasma was applied to the nanoparticles, a large amount of IgG was removed with no significant change in the concentrations of other plasma proteins which is also in correlation with previously reported studies (Yue et al.2008; Uygun et al., 2009). It must be pointed that nanoparticle sizes/body, functional groups, surface area and ligand loading lead to the differences in IgG Adsorption.
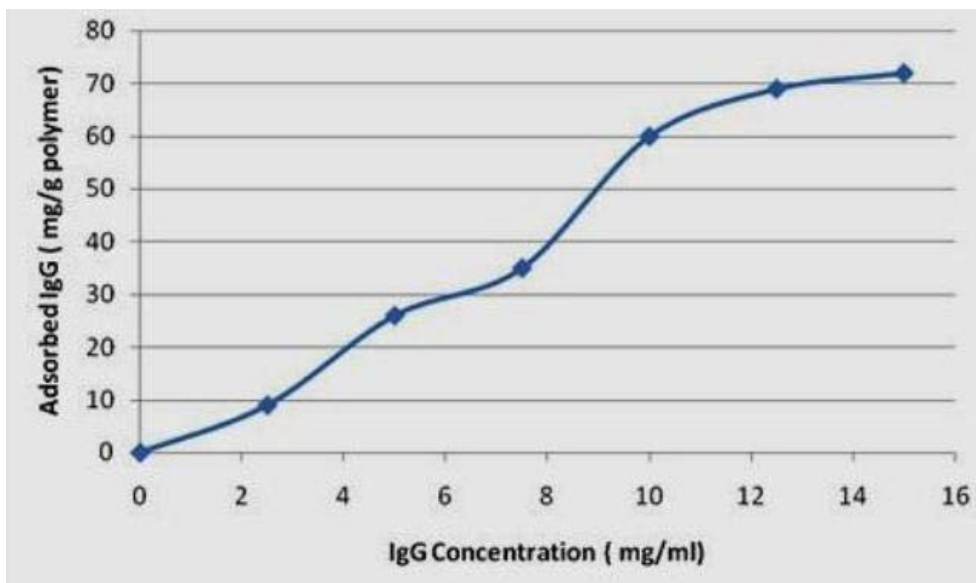
Fig. 8. IgG Adsorption from human plasma

4.7 Desorption of IgG and Reusability

The desorption of IgG was also studied. The final IgG concentration in the desorption medium was determined by UV spectroscopy. The desorption ratio was calculated from the amount of IgG adsorbed on nanoparticles and the final IgG concentration in the desorption medium. The reusability of the Con-A immobilized magnetic chitosan nanoparticles was tested by repeating the IgG adsorption–desorption cycle 5 times with the same particles. Fresh human plasma was used for repeated IgG adsorption cycles.
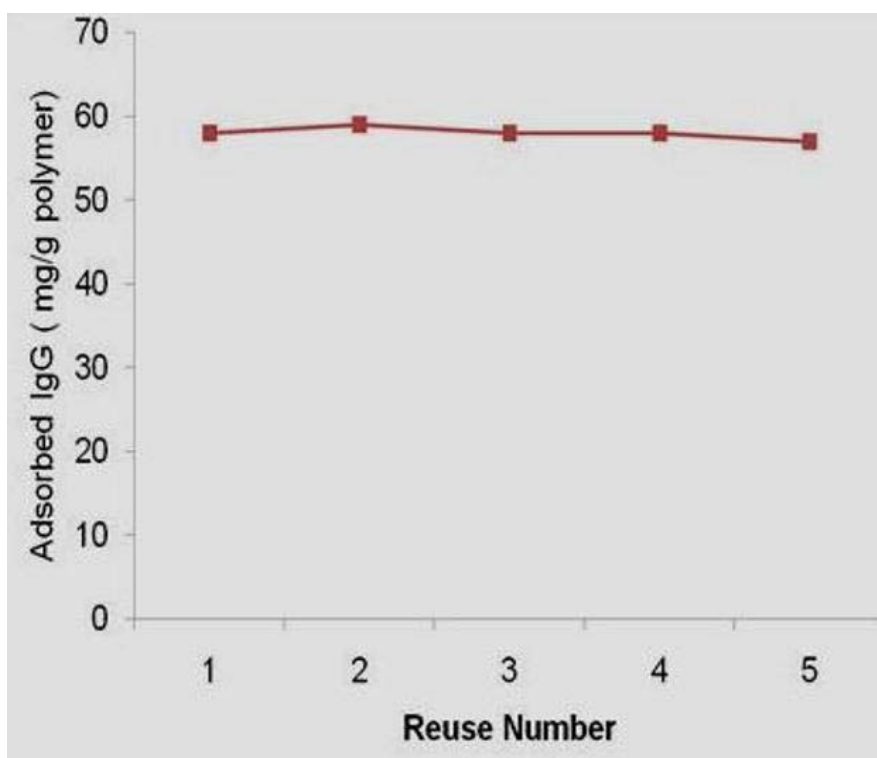

Fig. 9. Reusability of IgG adsorbed Con-A immobilized magnetic chitosan nanoparticles

Fig. 9 shows the reusability of the Con-A immobilized magnetic chitosan nanoparticles after 5 adsorption-desorption cycle. Please note that these cycles are completely reversible and the system shows no notable decrease in IgG adsorption capacity. The IgG adsorption capacity decreased nearly 1.0 -2.0% after 5 cycles.

5. Conclusion

In this present study; magnetic chitosan nanoparticles were prepared by co-precipitation technique. Well-dispersed Fe_3O_4 nanoparticles were formed during the formation of chitosan nanoparticles. Data shows that it is possible to change the nanoparticle size by changing the chitosan molecular weight and chitosan/TPP ratio during the preparation of the nanoparticles. Loading ratio of Con A was 70 mg/g and adsorption ratio of IgG was 63 mg/g from aqueous solutions. The maximum adsorption capacity was achieved in phosphate buffer system at pH 7.0 nearly to body reaction pH 7.4. Desorption ratio was >95% of adsorbed IgG which was achieved with 2.0 M NaCl. The results show that IgG adsorbed Con A attached Magnetic Chitosan Nanoparticles can be reused without a notable decrease in the adsorption capacities. Therefore, we demonstrate that due to characteristics such as strong specificity and excellent reusability, magnetic chitosan nanoparticles are promising and have great potential for the purification of antibodies such as IgG and other biomolecules.

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