Evaluation of Chitosan Tripolyphosphate Gel Beads as Bioadsorbents for Iron in Aqueous Solution and in Human Blood *In Vitro*

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ABSTRACT: Nonimprinted and Fe^{3+} imprinted chitosan tripolyphosphate gel beads were prepared via physical gel formation. A method based on *in situ* crosslinking using ethylene glycol diglycidyl ether was developed to imprint the chitosan tripolyphosphate gels with Fe^{3+} ion without deteriorating the gel beads. The beads were characterized by FTIR, SEM, XRD, and DSC with respect to the chemical structure, surface morphology, crystallinity, and thermal behavior. Swelling kinetics and Fe^{3+} ion adsorption behavior from aqueous solution were studied. The Fe^{3+} imprinted and *in situ* crosslinked beads proved to be durable and effective adsorbents for Fe^{3+} in solution. The bead prepared by *in situ* crosslinking and in the presence of 10 m*M* template ion had an equilibrium iron adsorption capacity of 53.9 mg/g after 3-hour contact with 5 m*M* Fe^{3+} solution. The pros and cons of the beads as biomedical iron adsorbents were tested by evaluating their serum iron removal capacities from human blood. The preliminary tests carried out showed that Fe^{3+} imprinted beads were more effective in decreasing serum iron in human blood when compared to the nonimprinted beads. The decrease in serum iron level accompanied a parallel decrease in the hemoglobin level. The calcium level was also affected upon contact with the beads. The Fe^{3+} imprinted beads were less effective than the nonimprinted ones in decreasing the calcium level indicating selectivity towards iron containing species. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 125: 1493–1505, 2012

Key words: crosslinking; molecular imprinting; hydrogels; adsorption; biopolymers

INTRODUCTION

Chitosan has long been known as a biocompatible, biodegradable, and nontoxic polymer with a relatively low cost whose chemical functionality allows its modification to several different derivatives with potential biomedical applications.[1,2] Although chitosan has an undeniable potential for biomedical applications, its chemical functionality that furnishes it with a range of physicochemical, biochemical and biological responses can sometimes turn to a disadvantage. The multifunctional character of chitosan such as having an affinity for metals, fats and proteins at the same time brings in a selectivity problem to be solved. Furthermore, degradation behavior, toxicity, and biodistribution of chitosan and its derivatives vary with degree of deacetylation, molecular weight, and particle size, the type of the side groups and presence of impurities.[3] Hence, it is of utmost importance to elucidate the biological responses and the biochemical changes induced by the chitosan based biomaterial along with the prime effect under investigation. Our initial studies have revealed that chitosan flakes could successfully adsorb free or citrate-sorbitol complexed Fe^{3+} from solution, and reduce ferritin level in the blood serum samples of thalassaemia patients.[4] In this study, chitosan tripolyphosphate gels were tested as bioadsorbents for Fe^{3+}. This type of beads is also known for its biocompatibility, a virtue needed for biomedical applications.[5] Chitosan tripolyphosphate gels have been prepared by using a technique similar to the ones described by various authors in the literature.[6–11] The basic principle behind the method is the physical gel formation via ionic cross linking between cationic chitosan and polyanions of the tripolyphosphate in solution. Factors that affect the physical properties of the gels prepared include the pH of the gelling medium and the concentrations of the gel-forming ions. Furthermore, an easy and reliable method was developed to prepare Fe^{3+} ion imprinted chitosan tripolyphosphate gels with a higher potential Fe^{3+} affinity. Although a number of articles are available in the literature describing the

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synthesis and applications of imprinted synthetic organic polymers,[12–15] those on imprinting of natural polymers is very limited.[16] Preparation of imprinted synthetic polymers basically involves polymerization of the monomer complexed to the template molecule or ion, in the presence of a crosslinker and a suitable porogen. After polymerization is complete, the template molecule and the excess monomer are removed from the system leaving behind an imprinted polymer with specific recognition sites. Imprinting of natural polymers with a given template involves no polymerization stage. The imprinting process needs to be achieved via crosslinking the polymer in the presence of the template followed by removal of the template and the excess crosslinker. Hemoglobin imprinted chitosan based beads,[17] Ni^{2+} ion imprinted chitosan tripolyphosphate beads[18] and their characterization has been described in the literature. In both of these studies, surface crosslinking of the chitosan beads was employed rather than carrying out imprinting in the presence of the crosslinker, as described in this article. Reports are available on the preparation of Fe^{3+} selective imprinted polymer adsorbents based on poly(HEMA) prepared by the classical imprinting technique and tested for analytical purposes.[19–22] No report could be found in the literature describing the preparation and characterization of Fe^{3+} imprinted chitosan resins or any other natural polymer.

This article gives an account of the preparation of nonimprinted and Fe^{3+} imprinted chitosan tripolyphosphate gel beads including the effect of the preparation conditions and the crosslinking method on the physical properties such as the surface morphology, swelling, and dissolution characteristics and on the Fe^{3+} adsorption behavior. The Fe^{3+} uptake capacities of the beads were evaluated both in aqueous solution and in human blood, *in vitro*. Accompanying changes in the hemoglobin, albumin, and calcium levels have also been reported.

EXPERIMENTAL

Following materials, instruments and methods were used to prepare and characterize the samples. In all blood analyses, the blood samples used were obtained from volunteers with their consent.

Materials

Chitosan flakes (Fluka) of molar mass 4.0×10^5 (MM_w) g/mol, and degree of deacetylation of 85% were used. Sodium hydroxide (Aldrich), acetic acid (Aldrich), hydrochloric acid (Merck), ferric chloride (Aldrich), sodium tripolyphosphate pentabasic (Aldrich), EGDE (ethylene diglycidyl ether) (Aldrich), 5-sulfosalicylic acid dihydrate (Riedel-de Häen), potassium chloride (BDH), and ammonia (Merck) were used without any purification. Buffer solutions used in these experiments were prepared using potassium chloride and hydrochloric acid.

Preparation of chitosan tripolyphosphate beads by post formation crosslinking and *in situ* crosslinking

Chitosan solution was prepared by dissolving chitosan flakes in 1% (v/v) acetic acid solution and was dropped into a coagulation bath consisting of a mixture of sodium tripolyphosphate pentabasic (TPP) in pH = 1.2 buffer to prepare nonimprinted chitosan tripolyphosphate beads.

To prepare post formation crosslinked nonimprinted beads, the beads formed were collected and were treated with EGDE. They were then washed with distilled water for neutralization and dried overnight at 60°C.

In situ crosslinked nonimprinted beads were formed by including EGDE solution in the TPP coagulation medium at pH 1.2. The beads were cleaned extensively as given above and dired overnight at 60°C.

Fe^{3+} ion was used as the template to prepare the imprinted chitosan beads. Two methods were applied. In the first method, chitosan in acetic acid solution was dropped into a coagulation bath consisting of a mixture of TPP in pH = 1.2 buffer and ferric chloride. The beads formed were collected and crosslinked by being treated with EGDE. Then, they were washed with distilled water and cleaned from Fe^{3+} ion using 0.06*M* NH_3 solution until the solution was colorless. The template free beads were washed with distilled water for neutralization. Finally, they were extensively washed with distilled water and dried overnight at 60°C.

In the second method, the *in situ* crosslinking, chitosan dissolved in acetic acid was dropped into a mixture of TPP and ferric chloride solution containing the crosslinker, EGDE. The template ion Fe^{3+} was removed and the beads obtained were washed and dried as given above. Average bead diameter was 1 mm.

Swelling and dissolution of the beads

The swelling behavior of the prepared beads was measured in pH = 1.2 at room temperature at 25°C. A buffer solution (100 mL) of pH = 1.2 was prepared by mixing 25 mL 0.2*M* KCl and 42.5 mL 0.2*M* HCl. Beads (~0.05 g) were immersed and kept in the buffer solution (pH 1.2) for 6 hours with stirring at 50 rpm at 37°C. The wet mass of the swollen gels were determined by blotting them with a filter paper to remove the surface water. They were then

immediately weighed on an electronic balance. The swelling ratio of the beads was calculated using eq. (1) where, m_{d_1} and m_s are the weights of the samples in the dry and swollen states, respectively. Each swelling experiment was repeated three times and the average value was taken.

$$\text{Swelling ratio}\,(\%) = \frac{m_s - m_{d_1}}{m_{d_1}} \times 100 \qquad (1)$$

The soluble fraction was determined by immersing and keeping a sample of hydrogel beads (~0.05 g) in the buffer solution (pH 1.2). The swollen hydrogel samples were taken and dried in oven at 60°C to constant weight. The soluble fraction was calculated according to the following equation:

$$\text{Weight Loss}\,(\%) = \left(\frac{m_{d_1} - m_{d_2}}{m_{d_1}}\right) \times 100 \qquad (2)$$

where m_{d_1} and m_{d_2} are the weights of dried hydrogel before and after contact with the buffer solution.

Adsorption of Fe^{3+} ion on chitosan tripolyphosphate beads

Fifty milligram bead sample was placed in a 50 mL aqueous Fe^{3+} solution at pH = 1.2 and stirred at 60 rpm at 37°C for 6 hours. One-milliliter aliquots were taken at 1-hour intervals and analyzed for Fe^{3+} concentration by visible spectrophotometry. The amount of Fe^{3+} adsorbed was calculated from the difference between the concentrations of the initial and final solution. All adsorption experiments were done at least in duplicate.

Determination of Fe^{3+} in solution

One milliliter Fe^{3+} solution was mixed with 1 mL of 5-sulfosalicylic acid dehydrate, (10% w/v), and completed to 10 mL with pH = 1 buffer solution. Twenty-five milliliter 0.2*M* KCl and 67.0 mL 0.2*M* HCl is used to prepare 100 mL of the pH = 1 buffer solution. The amount of residual Fe^{3+} ion was determined by visible spectrophotometry at 505 nm using a Shimadzu UV-1201 V visible spectrophotometer. Then the amount of Fe^{3+} ion adsorbed by chitosan was calculated.

Determination of serum iron

Ten milligram of beads were brought into contact with 500 μL of human serum for 3 hours *in vitro*. Total iron was measured using a commercial Ferrimat assay kit (bioMérieux, France). The amount of transferrin bound iron was determined by BTS 310 (4.0 ver) spectrophotometer at 562 nm. A calibration was performed for each series of tests.

Determination of hemoglobin in whole blood

Ten milligram of beads were brought into contact with 500 μL of blood for 3 hours *in vitro*. Medonic CA 530 (20 Parameter system Thor) equipment were used for hemoglobin analysis. The level of hemoglobin in the blood was measured before and after contact with the beads.

Determination of albumin in blood serum

Ten milligram of beads were brought into contact with 100 μL of human serum for 3 hours *in vitro*. Albumin test kit (MBT, Biomer) was used. Then, 1 mL of reagent, 5 μL of standard and 5 μL of serum were taken up and incubated for 10 min at 37°C. The amount of final albumin level was determined by BTS 310 (4.0 ver) spectrophotometer at 630 nm.

Determination of total calcium in blood serum

Ten milligram of beads were brought into contact with 100 μL of human serum for 3 hours *in vitro*. Calcium test kit (MBT, Biomer) were used. Then, 1 mL of reagent, 15 μL of standard and 15 μL of serum were taken up and incubated for 10 min at 37°C. The amount of final total calcium level was determined by BTS 310 (4.0 ver) spectrophotometer at 670 nm.

FTIR analysis

The FTIR spectra of chitosan tripolyphosphate beads were recorded at TUBITAK-MAM, Gebze with KBr pellets on a Perkin Elmer Spectrum One FTIR spectrometer.

SEM analysis

SEM analysis of the samples was carried out at TUBITAK-MAM, Gebze using a JEOL/JSM-633F scanning electron microscope.

XRD analysis

X-ray powder diffraction analysis were carried out for chitosan, N-SC8, I5-SC8, and N-PC8 at TUBITAK-MAM, Izmir by using a Phillips X'Pert Pro X-Ray powder diffractometer (wavelength = 1.54060 Å). The samples were exposed to the X-ray beam from X-ray generator running at 45 kV and 40 mA. The scanning regions of the diffraction angle 2θ were 5–80°, which covered most of the significant diffraction peaks of the chitosan crystallites. The

crystallinity indices were determined using eq. (3). I_{110} was read from $2\theta = 20^\circ$ and I_{am} from $2\theta = 8^\circ$.

$$\mathrm{CrI}\,\% = \frac{(I_{100} - I_{am})}{I_{110}} \times 100 \quad (3)$$

DSC analysis

Thermal behavior of the chitosan tripolyphosphate beads was kept at 105°C for 2 hours to remove the moisture of the samples prior to DSC measurements. Then, the samples were analyzed using a Perkin Elmer Jade calorimeter at TUBITAK-MAM, Gebze. The samples were studied under nitrogen atmosphere. The heating rate was 10°C/minute and the temperature range was 10–300°C.

RESULTS AND DISCUSSION

The swelling and dissolution properties and the Fe^{3+} adsorption behavior from aqueous solution were studied. The physicochemical properties of the Fe^{3+} imprinted and nonimprinted gel beads were compared to each other and were discussed in terms of chemical and physical properties. The potential application of the beads as bioadsorbents for iron containing substrates was tested upon contact with human blood, *in vitro*.

Bead preparation and bead morphology

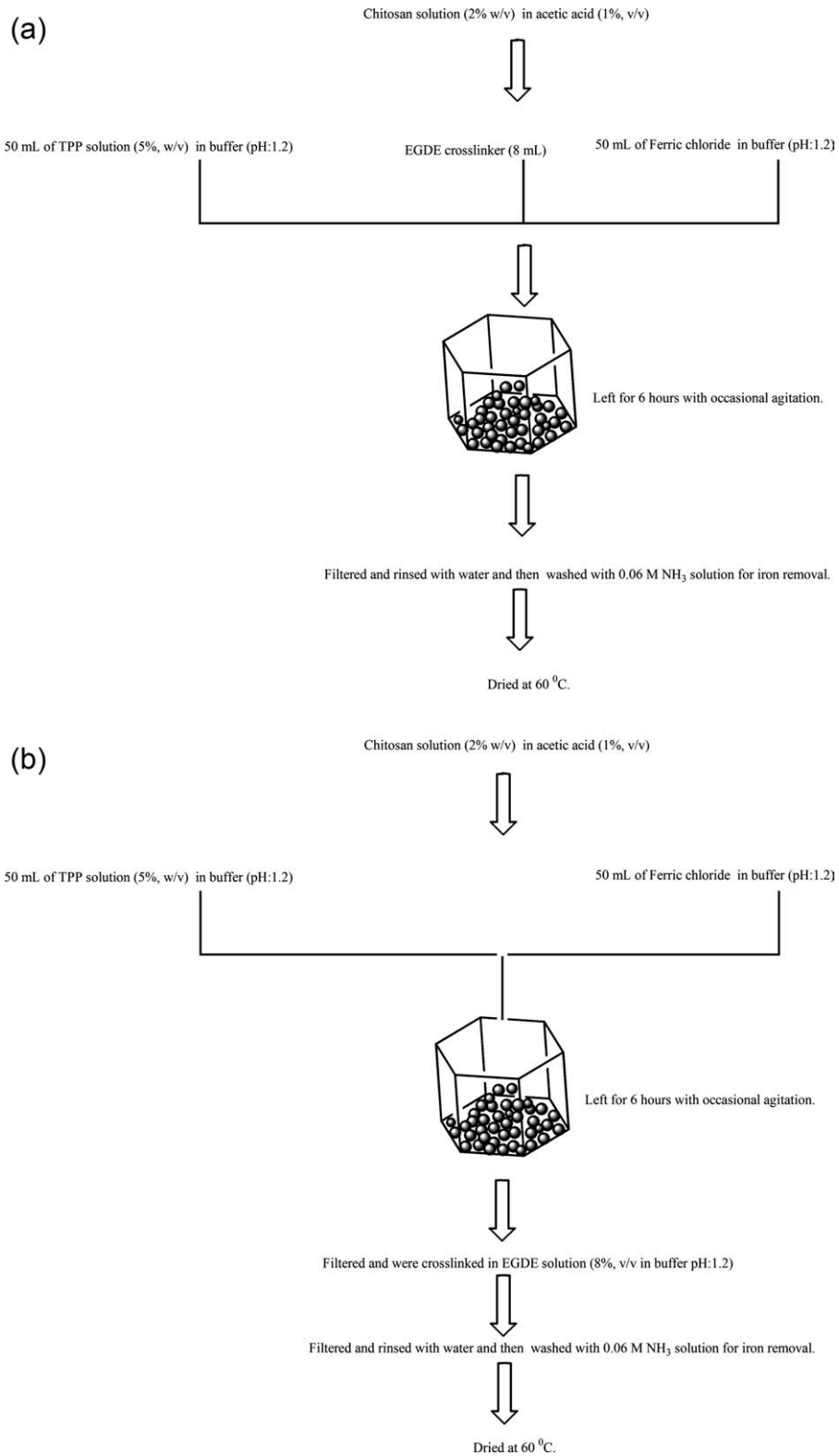
Chitosan tripolyphosphate beads were prepared under acidic conditions via ionic crosslinking between cationic chitosan macromolecule and tripolyphosphate ions available in the medium. Gel formation occurs due to the interaction of the polymer molecules with the polyfunctional anion leading to a three dimensional network structure. Spherical, homogeneous beads form instantaneously when the chitosan solution is dropped into the aqueous solution containing the tripolyphosphate ion. Chemical crosslinking was performed in two different ways; *In situ* crosslinking was achieved by including ethylene glycol diglycidyl ether (EGDE) in tripolyphosphate solution during bead formation [Scheme 1(a)]. In the second method, the post formation crosslinking, beads were treated with aqueous EGDE solution after they had been formed [Scheme 1(b)]. Ion imprinting was carried out by using Fe^{3+} ion as the template. Gel bead formation taking place in the presence of the Fe^{3+} ion allows inclusion of the ion in the network structure. It is known that chemical interaction between Fe^{3+} and chitosan occurs via amine and hydroxyl groups of chitosan and oxygen containing sites available in the medium.[23] In this case, the phosphate groups available in the medium should also take part in the chelation process.

Removal of the template ion from the bead leaves behind a Fe^{3+} imprinted network structure. The bead preparation conditions have been summarized in Table I.

The morphological features of the chitosan tripolyphosphate beads prepared via *in situ* crosslinking were studied by scanning electron microscopy (SEM) in the dry state. SEM micrograph of the samples N-SC8, I5-SC8, and I10-SC8 are shown in Figure 1(a–c), respectively. N-SC8, I5-SC8, and I10-SC8 were formed under similar experimental conditions, using a mixture of 5% TPP and 8% EGDE solution. I5-SC8 and I10-SC8 are Fe^{3+} imprinted samples prepared in the presence of 5 m*M* and 10 m*M* Fe^{3+} solution. N-C8 is the nonimprinted counterpart of these samples. All beads are spherical with a smooth, nonporous, and homogeneous surface as observed at low magnification (×45). The effect of imprinting on the morphology is clearly observable from the micrographs taken at high magnification (×2000). The surface of I10-SC8 is smoother than that of I5-SC8 and the surface of I5-SC8 is smoother than that of N-SC8. This observation indicates that complexation with the template ion Fe^{3+} brings polymer chains closer together during bead formation. Furthermore, the chemical treatment on the beads to remove the template ion does not disturb this compact structure formed.

FTIR analysis

FTIR spectra of N-PC8, I5-PC8, I5-SC8, and N-SC8 are given in Figure 2(a–d). In all chitosan tripolyphosphate beads, in 3410–3440 cm^{-1} region O—H stretchings and 2870–2890 cm^{-1} region —C—H— stretching vibrations are observed. The C=O stretching at 1735 cm^{-1} is due to the presence of acetate ion of chitosan acetate salt. At 1642–1648 cm^{-1}, amide I band of acetamido group of chitosan is observable. N—H bending of the prototonated amino group of chitosan is available at 1570 cm^{-1}. Since bead formation was carried out in acidic medium, tripolyphosphate ion is present in its highly protonated form, the tripolyphosphoric acid, hence P—O stretching vibrations of tripolyphosporic acid are observed at 1247 cm^{-1}. At 1200–900 cm^{-1} C—O stretching vibration of the pyranose ring overlap with P—O and P—O—H stretchings. Sample I5-PC8 does not exhibit any acetate stretching, showing that this group was removed during template ion removal by ammonia. Furthermore, P—O stretching at 1247 cm^{-1} has decreased to a great extent indicating that tripolyphosphoric acid component of the post formation crosslinked and imprinted gel beads was removed during ammonia treatment.



Scheme 1 (a) Imprinted bead fabrication by *in situ* crosslinking. (b) Imprinted bead fabrication by post formation crosslinking.

XRD analysis

The degree of crystallinity of I5-SC8, N-PC8, N-SC8, and chitosan was evaluated by X-ray diffraction method. The diffraction patterns of these samples are shown in Figure 3(a–d), respectively. Chitosan has a strong reflection at $2\theta = 20^\circ$ and $2\theta = 10^\circ$ well established in the literature.[24] The crystalline peak at

TABLE I
Preparation Conditions for Chitosan Tripolyphosphate Gel Beads (in 1% v/v Acetic Acid Solution Using 2% (w/v) Chitosan Solution in 5% (w/v) TPP Solution at pH = 1.2 Buffer)

Sample ID	$[Fe^{3+}]$	Method of crosslinking and EGDE concentration (v/v)
N	None	None
N-PC1	None	Post formation, 1%
N-PC4	None	Post formation, 4%
N-PC8	None	Post formation, 8%
N-SC8	None	*In situ, 8%*
I5	5 m*M*	–
I5-PC8	5 m*M*	Post formation, 8%
I5-SC8	5 m*M*	*In situ, 8%*
I10-SC8	10 m*M*	*In situ, 8%*

N, Nonimprinted ; I5, Imprinted with 5 m*M* Fe^{3+} solution, I10, imprinted with 10 m*M* Fe^{3+} solution, P, post-formation crosslinking, S, *in situ* crosslinking; C1, 1% EGDE solution, C4, 4% EGDE solution; C8, 8% EGDE solution.

$2\theta = 20^\circ$ peak becomes broader and weaker in chitosan gel beads. The second crystalline peak at $2\theta = 10^\circ$, cannot be observed in the samples. The crystallinity indices of N-SC8, I5-SC8, and N-PC8 were determined as, 52.6, 52.4, and 50%, respectively. Crystallinity index of chitosan was determined as 63.6%. XRD results clearly show that crosslinking and imprinting results in partial loss of crystallinity when compared to raw chitosan.

DSC analysis

The DSC thermograms of I5-PC8, N-PC8, N-SC8, N-PC1, and I5-SC8 beads are shown in Figure 4(a–e), respectively. Chitosan decomposes during heating with a sharp exothermic peak at 300°C.[25] The I5-SC8 has a decomposition peak at 247°C, and the N-SC8, N-PC8, NPC1, and I5-PC8 have decomposition peaks at 235, 249, 239, and 273°C, respectively.

DSC analysis reveals that all beads except for I5-PC8 have similar thermal decomposition temperatures around 240°C indicating that they all have a similar ratio of hydrogen bonded amines-to-protonated amines. Loss of hydrogen bonding in chitosan structure is responsible from the exothermic decomposition upon thermal treatment.[25] It can be inferred from the method of bead preparation that I5-PC8 bears a much higher fraction of hydrogen bonded amine groups than the others as explained below. During preparation of imprinted and post crosslinked samples like I5-PC8, amine groups interact with the template ion, Fe^{3+} during gel formation. After the gel is formed, the ammonia treatment serves a dual purpose; removal of the template ion and neutralization of the gel beads by reformation of free amine groups capable of hydrogen bonding. Hence, I5-PC8 has a much higher thermal decomposition temperature (273°C) than the others. The endotherms observed in the other samples at around 260 and 270°C can be attributed to the presence of weak ionic interactions between protonated amine groups of chitosan and the tripolyphosphate ions. The last peak around 280–290°C should be due to the chemical crosslinks established by the reaction of chitosan with EGDE. Sample N-PC1 cannot exhibit the peak in this region because of low degree of crosslinking. The results are summarized in Table II.

Physical and chemical characteristics of the chitosan gel beads

An account of the overall physical and chemical properties of the gel beads is given below to help to understand the correlation between the physicochemical characteristics and the swelling/dissolution and Fe^{3+} adsorption behavior in solution. Results of SEM, FTIR, XRD, and DSC analyses reveal that:

1. All beads have smooth, nonporous surfaces, with layers becoming closer in imprinted and/or crosslinked surfaces. Hence, physicochemical processes like swelling and ion adsorption should be taking place mainly on the bead surface. Diffusion of small molecules or ions into inner depth should be limited by the nonporous surface.
2. Gel beads are formed due to weak ionic interactions between protonated chitosan and tripolyphosphate ions available in the medium under acidic conditions, and chemical crosslinking between the free amines and the etheric end groups of EGDE. Strong ionic interactions are missing as the bead formation took place in acidic medium causing the tripolyphosphate ion be protonated to a great extent.
3. During imprinting Fe^{3+} ion interacts with amine and oxygen containing groups available in the medium. Ammonia treatment leaves behind imprinted gel beads with unprotonated amine groups.
4. Crystallinities of all samples are close to each other, hence swelling or Fe^{3+} adsorption should be interpreted independent of sample crystallinity.

Swelling and dissolution behavior

Swelling characteristics of the beads were studied in aqueous solution at pH = 1.2, the same pH as the adsorption experiments were carried out. The swelling behavior of the samples is shown in Figure 5. All beads exhibited similar swelling kinetics. They

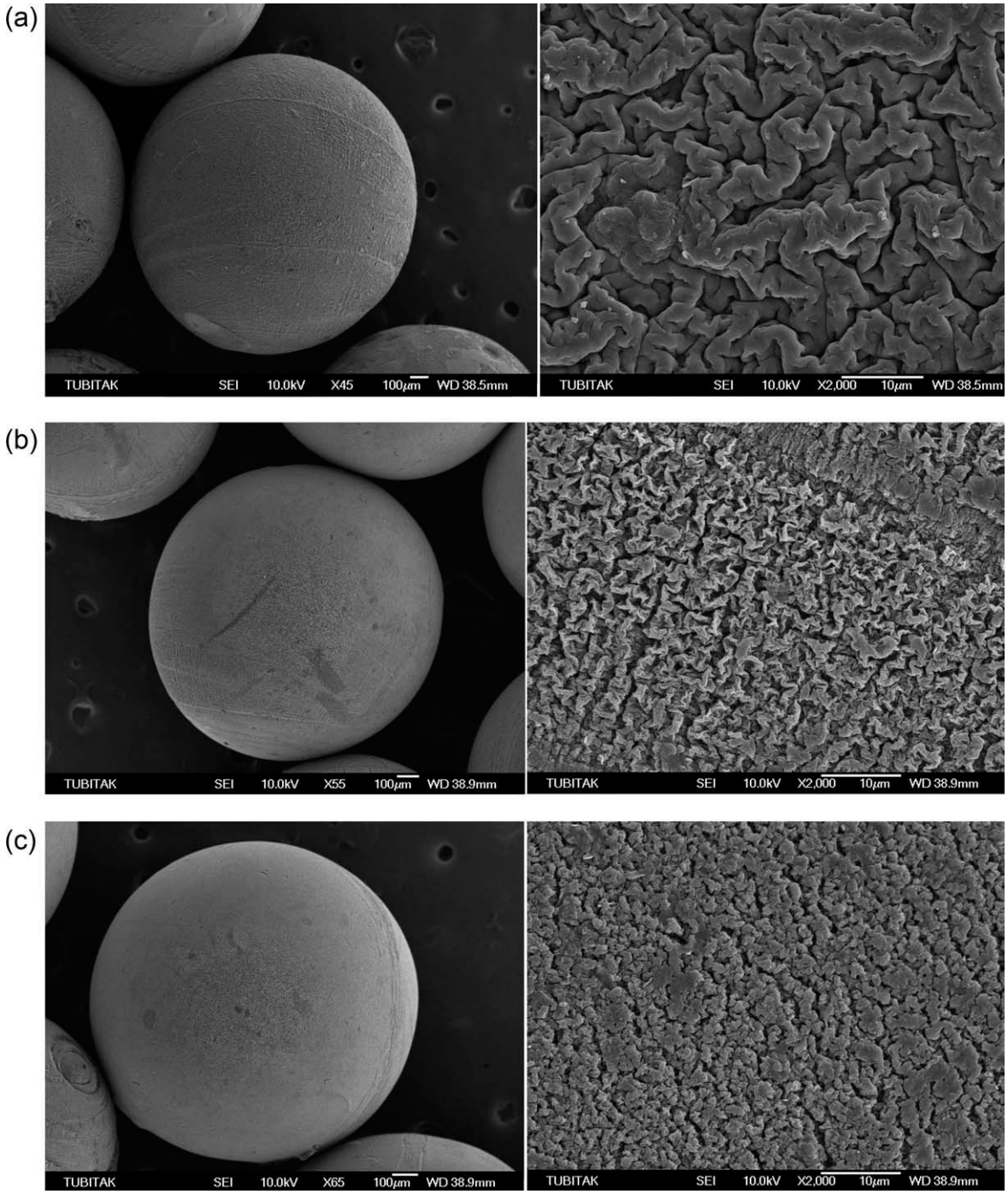


Figure 1 SEM micrographs with ×45 and ×2000 magnification (right) of (a) N-SC8, (b) I5-SC8, and (c) I10-SC8.

swelled and reached an equilibrium swelling capacity in time. Noncrosslinked samples as exemplified by samples N and I5 swell to dissolve within 6 hours time. Crosslinking decreases the swelling capacity but protects the samples from dissolving. Therefore, it is necessary to perform chemical crosslinking to be able obtain useful adsorbents. Increasing EGDE concentration used for the reaction, inversely affects the swelling capacity. This effect is illustrated for the nonimprinted samples that were crosslinked via post formation crosslinking. For example, N-PC1 swelled to an equilibrium value of 125%, N-PC4 to 62% and N-PC8 to 50%. Not only the degree of crosslinking but also the method of crosslinking affects swelling capacity. Sample N-SC8 prepared via *in situ* crosslinking using 8% EGDE solution, has an equilibrium swelling capacity of 104% which is higher than that of its counterpart prepared by post formation crosslinking, N-PC8. This sample has an equilibrium swelling capacity of 62%. Higher

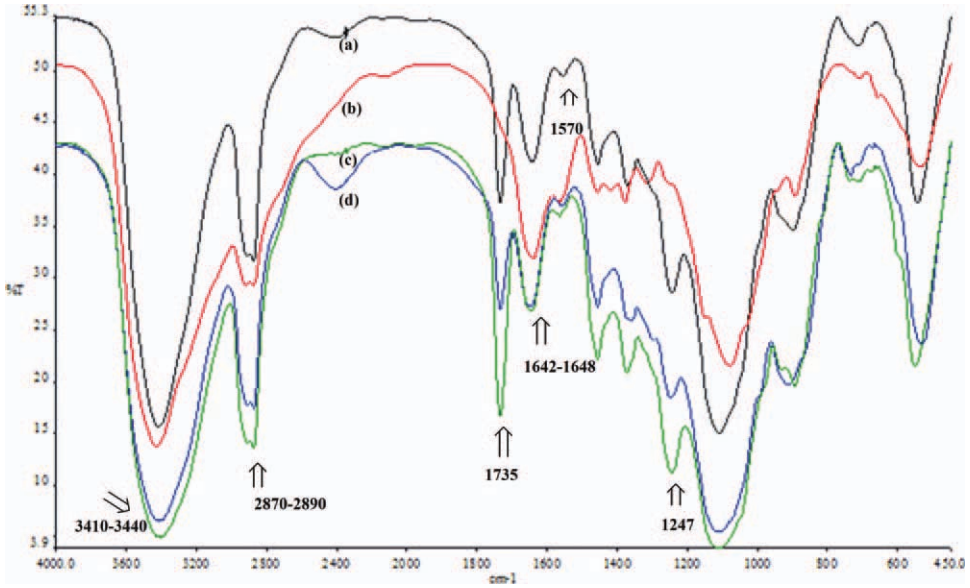


Figure 2 FTIR spectrum of (a) N-PC8, (b) I5-PC8, (c) I5-SC8, and (d) N-SC8. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

swelling capacity of the *in situ* crosslinked sample can be attributed to the chemical structure of the bead surface after crosslinking. Repulsion between the positively charged protonated amine groups is responsible from swelling taking place in acidic medium. During post formation, crosslinking amine groups on the bead surface are more susceptible to reaction with EGDE molecules. Hence, the fraction of free amine groups on the surface of this type of beads is less than that of the *in situ* crosslinked beads. Therefore, beads crosslinked by post formation crosslinking would bear less number of protonated amine groups in acidic medium causing less swelling [Scheme 2(a,b)]. *In situ* crosslinked beads, whether imprinted or not, were durable in aqueous medium for weeks without any measurable weight loss, while the post formation crosslinked ones disintegrated at the end of 24 hours showing that a weaker network with a lower crosslinking density was formed by post formation crosslinking.

Imprinting brings about a huge increase in the swelling capacity. Noncrosslinked but imprinted sample I5 has an equilibrium percentage swelling value of 1113. *In situ* crosslinked and imprinted beads I5-SC8 and I10-SC8 have equilibrium percentage swelling capacity values of 1152 and 1794%, respectively. It can be concluded that a strong chitosan-tripolyphosphate-EGDE network is formed during *in situ* crosslinking and the network could accommodate the template ion successfully via a

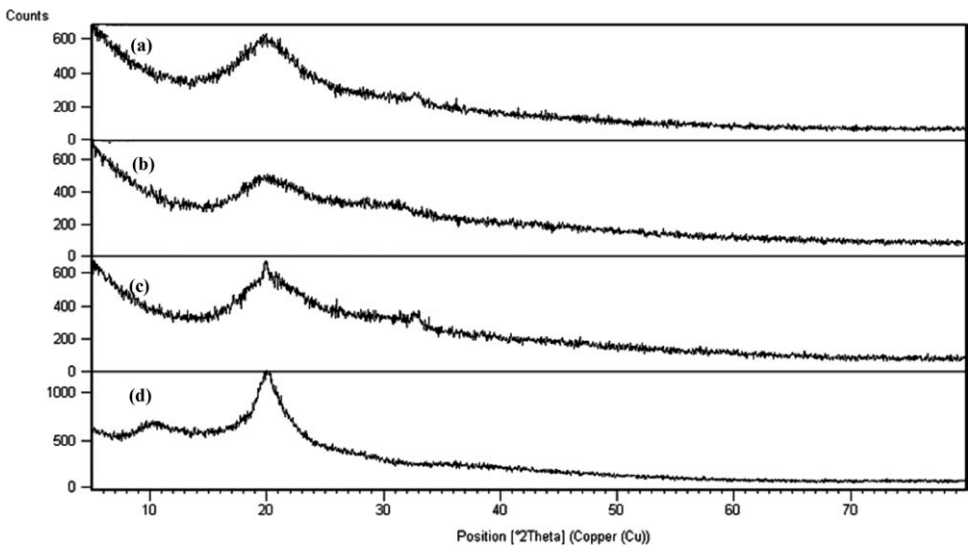


Figure 3 XRD pattern of (a) I5-SC8, (b) N-PC8, (c) N-SC8, and (d) chitosan.

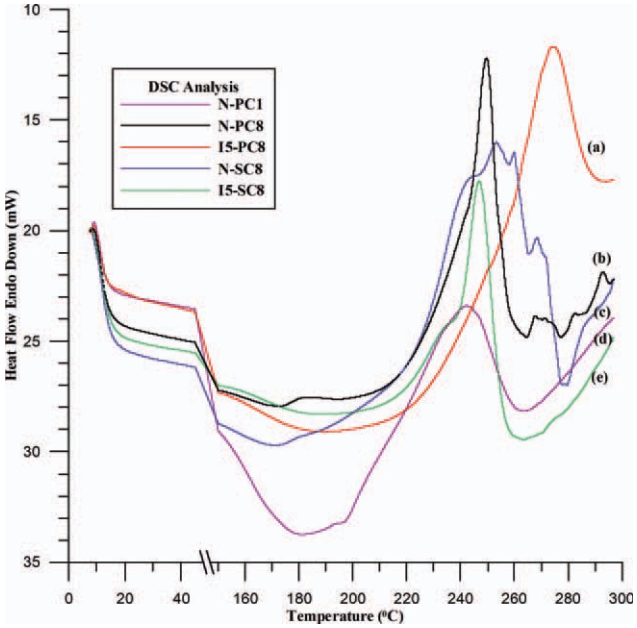


Figure 4 DSC thermogram of (a) I5-PC8, (b) N-PC8, (c) N-SC8, (d) N-PC1, and (e) I5-SC8. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

complex formation. Furthermore, the ammonia treatment applied to remove the template ion did not disturb the chitosan-tripolyphosphate-EGDE network formed but resulted in neutralization of the protonated amine groups. Hence, these beads bear free amine groups in the dry state. As discussed above, swelling depends primarily on the repulsion between protonated amine groups. As the imprinted beads bear a higher fraction of free amine groups in the dry state, they have a higher potential to swell via protonation of these free amine groups in the acidic swelling medium [Scheme 2(c,d)].

Fe^{3+} adsorption behavior

SEM analysis given above revealed a smooth, homogeneous, and nonporous surface for the chitosan tripolyphosphate beads (Fig. 1). The surface is even smoother, and more compact for the Fe^{3+} imprinted samples. This finding indicates that Fe^{3+} adsorption occurs mainly on the bead surface as lack of pores limits diffusion of ions and molecules into inner depths.

The Fe^{3+} ion adsorption behavior of chitosan tripolyphosphate beads prepared under various conditions was followed in aqueous solution. The dependence of the Fe^{3+} adsorption capacity on solute concentration and on the structural characteristics of the beads such as crosslinking and imprinting was studied. The adsorption capacity values determined for each sample are listed in Table III.

TABLE II
DSC Analysis

Sample ID	Exotherm (°C)	Endotherm (°C)
Chitosan	300[25]	–
I5-PC8	273	–
N-PC8	249	260, 275, 290
N-PC1	239	262 (Broad)
I5-SC8	235	260 (Broad), 270(Shoulder), 282 (Shoulder)
N-SC8	235	258, 267, 278

It can be followed from Table III that all chitosan tripolyphosphate beads studied have higher equilibrium adsorption capacity values towards Fe^{3+} in 5 m*M* solution than in 1 m*M* solution. A higher concentration gradient results in higher adsorption from solution. Adsorption capacity is limited by the number of available sites for chelation.

The equilibrium adsorption capacities of N-PC1, N-PC4, and N-PC8 are 30.7, 21.7, and 19.8 mg/g showing a tendency to decrease with increasing EGDE concentration in the medium since the crosslinking reaction between EGDE and amino groups of chitosan results in a decrease in the fraction of free amino groups on the surface [Scheme 2(a)]. Fe^{3+}

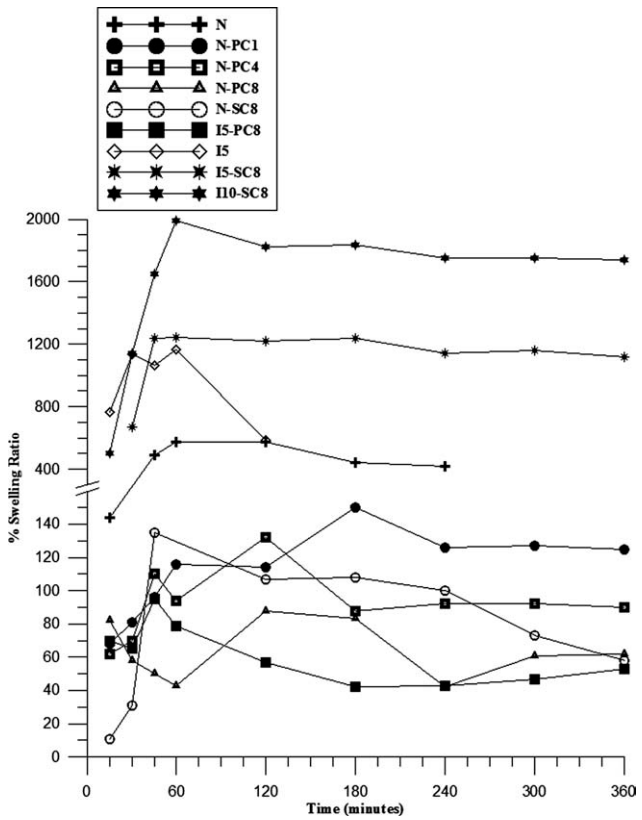
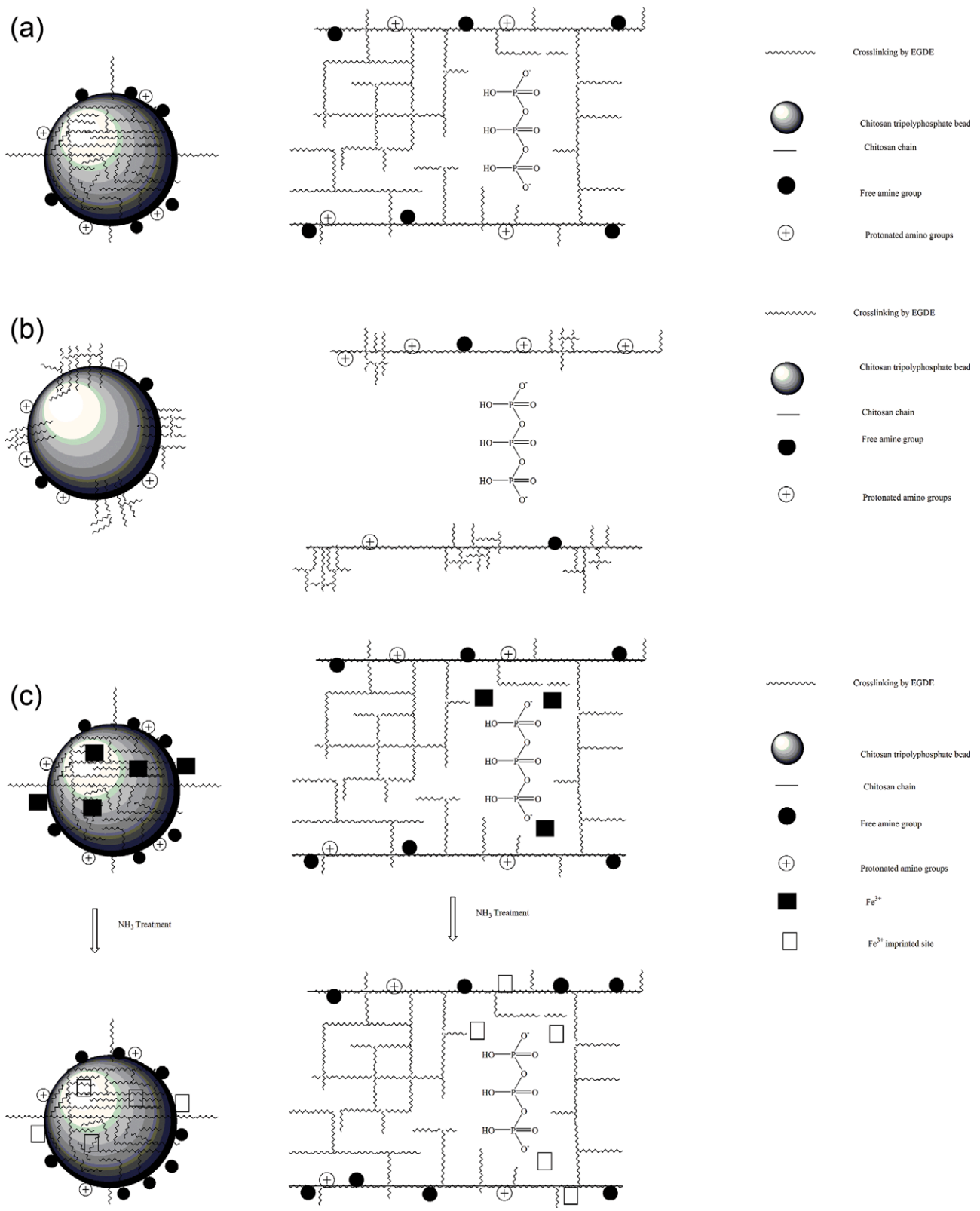
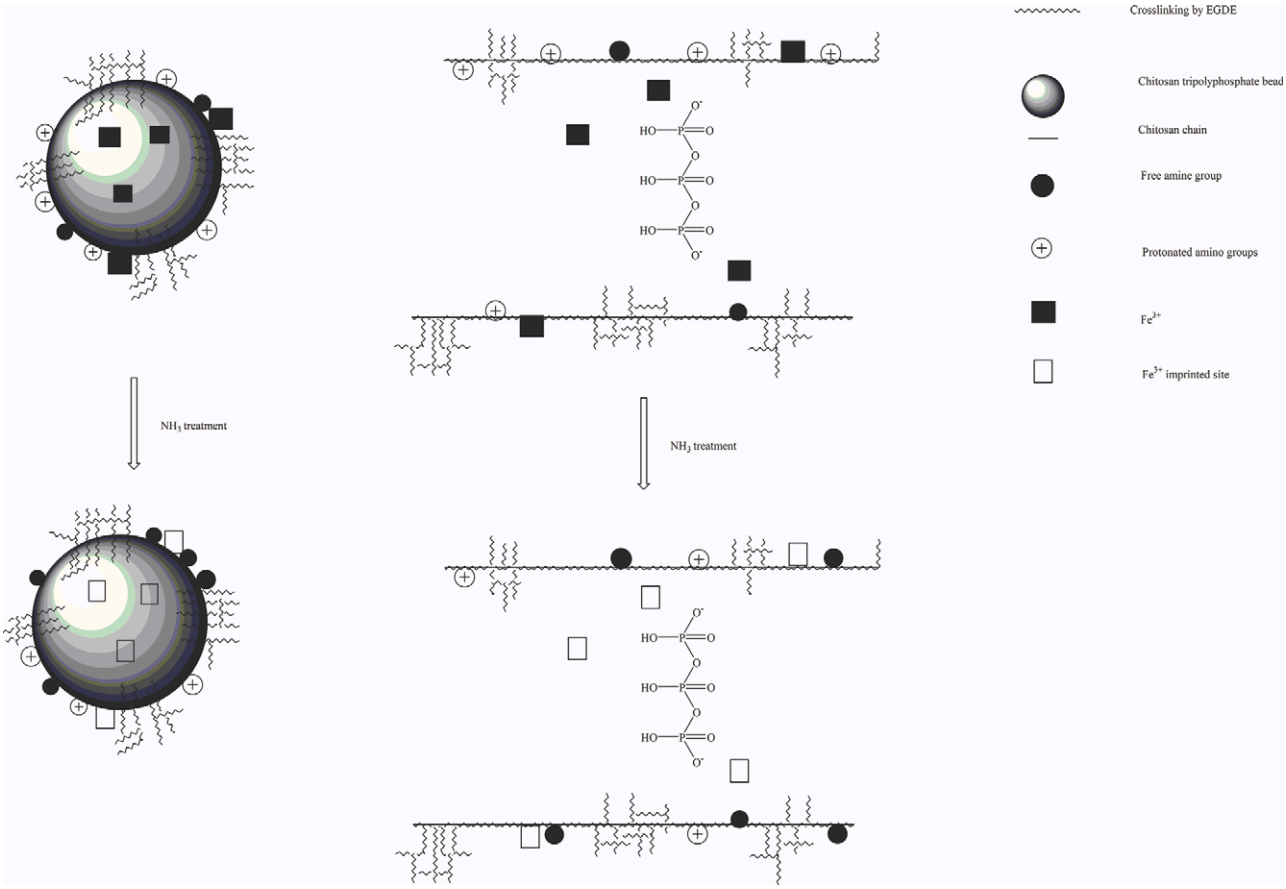


Figure 5 The swelling characteristics of the beads (N-PC1, N-PC4, N-PC8, I5-PC8, N-SC8, I5, I5-SC8, and I10-SC8) in aqueous solution at pH = 1.2.



Scheme 2 (a) The sketch of nonimprinted beads by *in situ* crosslinking. (b) The sketch of nonimprinted beads by post formation crosslinking. (c) The sketch of imprinted beads by *in situ* crosslinking. (d) The sketch of imprinted beads by post formation crosslinking. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



Scheme 2 (Continued)

adsorption capacity of the post formation crosslinked and Fe^{3+} imprinted and nonimprinted chitosan tripolyphosphate beads was compared to each other to follow the effect of Fe^{3+} imprinting on the adsorption capacity. Adsorption capacity values of nonimprinted and Fe^{3+} imprinted chitosan tripolyphosphate beads in 1 m*M* and 5 m*M* $FeCl_3$ solution are shown in Table III. The imprinted sample, I5-PC8 has a lower equilibrium adsorption capacity (13.1 mg/g) than its nonimprinted counterpart, N-PC8. Even though imprinted sites are available on the surface of I5-PC8, amine groups have taken part in the crosslinking reaction on the surface. Hence, they cannot participate in iron chelation. The *in situ* crosslinked counterpart (I5-SC8), however, is much more effective (39.5 mg/g) than I5-PC8 or N-PC8. The reason lies behind the fact that crosslinking has occurred homogeneously during bead formation. Specific loss of amine groups on the surface to crosslinking has been avoided. Hence, chelation of Fe^{3+} on the surface may occur more effectively leading to a higher adsorption capacity. The favorable effect of imprinting is more pronounced in the case of I10-SC-8, which has an equilibrium adsorption capacity of 53.9 mg/g.

Iron removal in human blood *in vitro* and a short account of accompanying changes in the blood composition

It is known that about 60% of the iron in human body is found in hemoglobin, 5% in myoglobin. The rest 35% is stored as ferritin or hemosiderin. A very small amount is transported between the body

TABLE III
The Swelling and Fe^{3+} Adsorption Capacities at Equilibrium for Prepared Imprinted Chitosan Gel Beads (in 1% v/v Acetic Acid Solution Using 2% (w/v) Chitosan Solution in TPP Dissolved at pH = 1.2 buffer)

Sample ID	Equilibrium swelling (%)	Equilibrium Fe^{3+} adsorption capacity (mg)/g chitosan ± Standard deviation	
		1 m*M* $FeCl_3$	5 m*M* $FeCl_3$
N-PC1	125	24.6 ± 0.283	30.7 ± 0.0354
N-PC4	90	20.5 ± 0.0566	21.7 ± 0.212
N-PC8	62	12.9 ± 0.424	19.8 ± 0.184
I5-PC8	50	10.2 ± 0.198	13.1 ± 0.00141
N-SC8	104	17.4 ± 0.0283	39.1 ± 0.0989
I5	1113	9.5 ± 0.0566	31.2 ± 0.0141
I5-SC8	1152	15.9 ± 0.141	39.5 ± 0.282
I10-SC8	1794	17.1 ± 0.0707	53.9 ± 0.0424

TABLE IV
Hemoglobin and Serum Iron Level Changes in the Blood Samples of Healthy Volunteers After 3 Hours of Contact *In Vitro* (10 mg of Beads With 500 µL Blood)

Initial Hemoglobin and iron level	Final Hemoglobin and iron level (% decrease)			
	N-SC8	I5-SC8	I10-SC8	N-PC8
Sample 1				
Hemoglobin (14.8 g/dL)	12.0, (18.9%)	3.5 (76.4%)	2.9, (80.4%)	9.7, (34.5%)
Iron (74 µg/dL)	59.0, (20.3%)	17.0, (77.0%)	14.5, (80.4%)	48.0, (35.1%)
Sample 2				
Hemoglobin (10.6 g/dL)	7.6, (28.3%)	5.8, (45.3%)	4.2, (60.4%)	7.2, (32.1%)
Iron (53 µg/dL)	38.0, (28.3%)	28.0, (47.2%)	20.5, (61.3%)	36.0, (32.1%)

compartments as bound to transferrin. There is a balance between the iron containing species in the blood. In cases of deficiency of iron or iron overload a complex set of biochemical reactions are triggered to overcome the anomaly. Iron removal from blood samples of healthy volunteers was studied by following the serum iron content in the blood serum, which is a measure of the transferrin-bound iron. The results are shown in Table IV. Same amounts of *in situ* crosslinked beads (N-SC8, I5-SC8, and I10-SC8) and post formation crosslinked beads (N-PC8) was brought into contact with the 500 µL of blood samples for 3 hours. The general behavior observed was that the imprinting had a significant effect on the iron removal capacity. The *in situ* crosslinked but nonimprinted beads (N-SC8) removed only 15 µg/dL in blood sample of sample 1, which corresponds to 20.3% decrease. The *in situ* crosslinked and Fe^{3+} imprinted beads I5-SC8 and I10-SC8, however, decreased the serum iron level from 74 to 17 µg/dL, and to 14.5 µg/dL, respectively. These values correspond to 77.0 and 80.4% decrease in the serum iron level. Sample N-PC8 decreased the serum iron level by 35.1%. A similar trend was observed in sample 2 as shown in Table IV. N-SC8, I5-SC8, I10-SC8, and N-PC8 induced a 28.3, 47.2, 61.3, and 31.2% decrease in the serum iron level, respectively.

One important effect accompanying iron uptake in blood was the change induced on the hemoglobin level. The beads were not only effective in reducing the serum iron level but they also induced a parallel decrease in the level of the iron containing protein hemoglobin as shown in Table IV. It can be followed that the trend observed for the serum iron removal is valid for hemoglobin level as well. Each sample induced an identical change in the hemoglobin level as it induced for the serum iron level as can be followed in Table IV. In sample 1, contact with N-SC8 results in a 20.3% decrease in the hemoglobin level as compared to 18.9% decrease in the serum iron level. Similarly, I4-SC8, I10-SC8, and N-PC8 induce a 77.0, 80.4, and 34.5% decrease in the hemoglobin level, respectively, which are all comparable to the serum iron level changes observed as 77.0, 80.4, and 35.1%, respectively. A similar effect is true for the sample 2 as well.

Hence, it can be deduced from the above given results that when iron present in blood is removed upon contact with the chitosan tripolyphosphate beads, the iron balance is disturbed. This disturbance is reflected as a decrease in the serum iron level. Further detailed analysis is needed to understand how iron metabolism is affected upon contact with the chitosan tripolyphosphate beads.

Contrary to the decrease in hemoglobin level upon contact with chitosan tripolyphosphate beads with human blood, a higher reading of free albumin level was obtained. Chitosan is known to have an affinity towards albumin. Hence, a decrease in free albumin level would have been the expected behavior. It can be followed from Table V that upon contact with the beads, an interdependent calcium : albumin level disturbance occurs in the most unexpected way. While this was only a slight change in the case of the Fe^{3+} imprinted beads (I10-SC8), it was more pronounced for the nonimprinted ones (N-SC8). In the first blood sample, there is deceptive increase in the albumin level from 58.7 to 73.2 and 60.2 g/L upon contact with N-SC8 and I10-SC8, respectively. Similarly, in blood samples of sample 2 and 3, the albumin level apparently increases from 81.2 to 95.1 and 93.8 g/L and from 82.4 to 92.3 and 83.6 g/L upon contact with nonimprinted and imprinted

TABLE V
Albumin and Total Calcium Level Changes in the Blood Serums of Healthy Volunteers After 3 Hours of Contact *In Vitro* (10 mg of Beads With 100 µL Blood)

	N-SC8	I10-SC8
Sample 1		
Albumin: 58.7 g/L	73.2 g/L	60.2 g/L
Total Calcium: 4.0 mg/dL	0 mg/dL	1.9 mg/dL
Sample 2		
Albumin: 81.2 g/L	95.1 g/L	93.8 g/L
Total Calcium: 7.5 mg/dL	0 mg/dL	6 mg/dL
Sample 3		
Albumin: 82.4 g/L	92.3 g/L	83.6 g/L
Total Calcium: 15.2 mg/dL	0 mg/dL	3.6 mg/dL

samples, respectively. As the total albumin content in blood should remain constant, this change should be due to the detection of free albumin unbound from other biochemical entities to which it was previously bound. This behavior can be explained by looking at the calcium level changes. Chitosan has an affinity towards calcium and it is known that calcium and albumin levels are interdependent as albumin is the carrier protein for calcium. It can be observed from Table V that the nonimprinted sample (N-SC8) adsorbs all calcium in all three-blood samples studied. The imprinted sample ((I10-SC8), however, adsorbs only a fraction of calcium present in the blood sample. When calcium complexed to albumin is transferred to chitosan gel beads, the protein albumin is set free, and hence an increase in the albumin level is recorded. The fact that the imprinted beads do not adsorb all calcium as the nonimprinted ones is a good indication of their selectivity towards iron bearing molecules in the blood.

Although, it is not clear whether it is the iron atom or it is the proteins and other iron carrying molecules that adsorb onto the beads, the fact that iron imprinted beads have higher iron removal capacities and lower calcium affinities than the nonimprinted ones indicates that iron atom present in the iron containing entities should have a role in the adsorption process.

It is also needed to understand if (i) changes other than iron, hemoglobin, and albumin levels such as cholesterol level are induced in the blood composition upon contact with the chitosan based beads and (ii) if the Fe^{3+} imprinted beads would selectively reduce iron in the case of high free and/or stored iron concentrations, that is, iron overload. More widespread analysis using simulated blood samples and a higher number of real samples is needed to be able to generalize these results and understand the chitosan-based biomaterial-blood interactions.

CONCLUSIONS

A practical and efficient method was developed to prepare Fe^{3+} imprinted chitosan gels by *in situ* crosslinking. The method can appropriately be modified for imprinting other ions on chitosan. All chitosan gel beads prepared were spherical with a smooth, nonporous and homogeneous surface. Fe^{3+} imprinting resulted in smoother bead surface indicating that complexation with the template ion Fe^{3+} brings polymer chains closer together during bead formation. The chemical treatment carried out on the beads to remove the template ion does not disturb this compact structure formed via *in situ* crosslinking. This method can be modified to imprint other template molecules to chitosan to obtain more effective and selective bioadsorbents. The beads proved to be effective bioadsorbents for iron in human blood as well as for hemoglobin. Iron imprinting increases affinity and selectivity towards iron containing species analyzed. A more detailed biochemical analysis is needed to understand and explain the blood-chitosan tripolyphosphate interactions.

References

1. Pillai, C. K. S.; Paul, W.; Sharma, C. P. Prog Polym Sci 2009, 34, 641.
2. Rinaudo, M. Prog Polym Sci 2006, 31, 603.
3. Kean, T.; Thanou, M. Adv Drug Delivery Rev 2010, 62, 3.
4. Burke, A.; Yilmaz, E.; Hasirci, N.; Yilmaz, O. J Appl Polym Sci 2002, 84, 1185.
5. Durkut, S.; Elcin, Y. M.; Elcin, A. E. ABB 2006, 34, 263.
6. Lee, S. T.; Mi, F. L.; Shen, Y. J.; Shyu, S. S. Polymer 2001, 42, 1879.
7. Lin, W. C.; Yu, D. G.; Yang, M. C. Colloids Surf B 2005, 44, 143.
8. Mi, F. L.; Shyu, S. S.; Kuan, C. Y.; Lee, S. T.; Lu, K. T.; Jang, S. F. J Appl Polym Sci 1999, 74, 1868.
9. Shu, X. Z.; Zhu, K. J. Int J Pharm 2002, 233, 217.
10. Shu, X.; Zhu, K. J. Int J Pharm 2000, 201, 51.
11. Mi, F. L.; Shyu, S. S.; Lee, S. T.; Wong, T. B. J Polym Sci Part B: Polym Phys 1999, 37, 1551.
12. Bergmann, N. M.; Peppas, N. A. Prog Polym Sci 2008, 33, 271.
13. Oral, E.; Peppas, N. A. J Biomed Mater Res 2004, 68A, 439.
14. Schmidt, R. H.; Belmont, A. S.; Haupt, K. Anal Chim Acta 2005, 542, 118.
15. Whitcombe, M. J.; Vulfson, E. N. Adv Mater 2001, 13, 467.
16. Yoshikawa, M.; Izumi, J.; Kitao, T. React Func Polym 1999, 42, 93.
17. Guo, T. Y.; Xia, Y. Q.; Wang, J.; Song, M. D.; Zhang, B. H. Biomaterials 2005, 26, 5737.
18. Su, H. J.; Chen, S.; Tan, T. W. Process Biochem 2007, 42, 612.
19. Ersoz, A.; Denizli, A.; Ozcan, A.; Say, R. Biosens Bioelectron 2005, 20, 2197.
20. Saatcilar, O.; Satiroglu, N.; Say, R.; Bektas, S.; Denizli, A. J Appl Polym Sci 2006, 101, 3520.
21. Yavuz, H.; Arica, Y.; Denizli, A. J Appl Polym Sci 2001, 82, 186.
22. Yavuz, H.; Say, R.; Denizli, A. Mater Sci Eng C 2005, 25, 521.
23. Bhatia, S. C.; Ravi, N. Biomacromolecules 2000, 1, 413.
24. Jaworska, M.; Sakurai, K.; Gaudon, P.; Guibal, E. Polym Int 2003, 52, 198.
25. Yilmaz, E.; Adali, T.; Yilmaz, O.; Bengisu, M. React Funct Polym 2007, 67, 10.