

pH sensitive *N*-succinyl chitosan grafted polyacrylamide hydrogel for oral insulin delivery

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ABSTRACT

pH sensitive PAA/S-chitosan hydrogel was prepared using ammonium persulfate (APS) as an initiator and methylenebisacrylamide (MBA) as a crosslinker for oral insulin delivery. The synthesized copolymer was characterized by Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) study; morphology was observed under scanning electron microscope (SEM). The PAA/S-chitosan with ~38% of insulin loading efficiency (LE) and ~76% of insulin encapsulation efficiency (EE), showed excellent pH sensitivity, retaining ~26% of encapsulated insulin in acidic stomach pH 1.2 and releasing of ~98% of insulin in the intestine (pH 7.4), providing a prolonged attachment with the intestinal tissue. The oral administration of insulin loaded PAA/S-chitosan hydrogel was successful in lowering the blood glucose level of diabetic mice. The bioavailability of insulin was ~4.43%. Furthermore, no lethality or toxicity was documented after its peroral administration. Thus, PAA/S-chitosan hydrogel could serve as a promising oral insulin carrier in future.

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1. Introduction

Parenteral invasive insulin delivery, being the most common in diabetic treatment, demands more pliable administration routes due to its poor patient complaisance. For this, several alternative delivery routes have been investigated (Lassmann-Vague & Raccach, 2006). One being oral route administration, favors better patient consent however, multiple physicochemical barriers (enzymatic degradation, tight junction, mucous lining, etc.) in gastrointestinal (GI) tract, limits its applicability. To combat such barriers, different polymeric vehicles have been investigated in enhancing efficiency in oral route administration (Mukhopadhyaya, Mishra, Rana, & Kundu, 2012).

Hydrogels available in several physical forms (Hoare & Kohane, 2008) have myriad applications in biomedical field (Lowman, Morishita, Kajita, Nagai, & Peppas, 1999; Nakamura et al., 2004), tissue engineering, diagnostics, regenerative medicine, drug or protein delivery system, etc. (Caykara & Ilkay, 2006). Polymeric hydrogels can significantly absorb large quantities of aqueous materials and able to efficiently retain them even after using some pressure on it (Qiu & Park, 2001).

Both natural and synthetic polymers are used for hydrogel synthesis. Chitosan (natural polymer) has gained considerable attention in drug delivery due to its biodegradable, biocompatible, low-toxic, non-immunogenic and mucoadhesive properties (Nagpal, Singh, & Mishra, 2010). Chitosan isolated from chitin by alkaline hydrolysis, is the linear and partly acetylated (1→4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977; Muzzarelli et al., 2012). Different functional groups (amino and hydroxyl groups) on the structure make chitosan chemically active, facilitating effective encapsulation of several bio-molecules (proteins, drugs). But, poor water solubility restricts the wide application of chitosan in the biomedical field. A variety of chitosan derivatives have been synthesized such as PEGylated chitosan (Jiang et al., 2006), quaternized chitosan (Thanou, Florea, Geldof, Junginger, & Borchard, 2002), and trimethylated chitosan (Kean, Roth, & Thanou, 2005) to improve its solubility.

Water soluble chitosan derivatives like carboxymethyl chitosan based polyampholyte hydrogel system facilitated the protein delivery (Chen, Tian, & Du, 2004). Again, a novel pH-sensitive chitosan-g-poly(acrylic acid)/attapulgit/sodium alginate composite hydrogel bead for controlled drug delivery was proposed by (Wang, Zhang, & Wang, 2009) and *N*-succinyl chitosan was reported as a novel permeation enhancer for the intranasal absorption of isosorbide dinitrate (Na, Wang, Wang, & Mao, 2013). Here, we prepare succinyl chitosan (S-chitosan), pK_a 4.48 ± 0.2 , by the

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introduction of succinyl groups at the N-position of the glucosamine units of chitosan to improve its water solubility and pH sensitivity.

In the present work, the efficiency of S-chitosan grafted polyacrylamide hydrogel (PAA/S-chitosan) is investigated for successful oral insulin delivery. Polyacrylamide is grafted only to increase the number of amino groups in the hydrogel in order to encapsulate more insulin within it. Mainly, we aim to prepare a pH sensitive hydrogel for sustained oral insulin delivery, producing insulin bioavailability and no toxicity within the animal body. A comparative study was conducted to investigate the better potential of PAA/S-chitosan over native chitosan grafted polyacrylamide hydrogel (PAA-chitosan). The insulin loading, encapsulation efficiency, swelling behavior, release pattern in simulated gastric and intestinal buffer and mucoadhesion were examined. The morphology was observed under SEM and finally, *in vivo* hypoglycemic effects and toxicity study were commenced.

2. Materials and methods

2.1. Materials

Chitosan, molecular weight (MW) 365 kDa, degree of deacetylation (DDA) 78% was obtained from Himedia (India). White crystalline type potassium bromide (KBr) was purchased from Merck (India). Succinic anhydride, dimethyl sulfoxide (DMSO), ammonium persulfate (APS) were purchased from Sisco Research Laboratories (SRL), India. Acrylamide (AA), methylenbisacrylamide (MBA) were obtained from Merck (India). Insulin (Bovine insulin, 27USP units per mg) and alloxan monohydrate were purchased from Sigma–Aldrich. Mucin was purchased from (Himedia, India). Bovine insulin ELISA kit from LILAC Medicare Pvt. Ltd., serum glutamate pyruvate transaminase (SGPT), ALAT(GPT)-LS kit and serum glutamate oxaloacetate transaminase (SGOT), AST(GOT)-LS kit from Piramal Health Care Limited, Mumbai, India, lactate dehydrogenase LDH (P-L) kit and micro-protein kit from Crest Biosystems, Goa, India, creatinine merckotest kit (Merck Limited, Mumbai, India) were purchased. Multistix reagent strips (Siemens, Baroda, India) were used for purchased urine biochemical parameter analysis. Other chemicals of analytical grade were used.

2.2. Animals

Male Swiss albino mice (26 ± 2 g), (from M/s Chakraborty Enterprise, Calcutta, India) were housed under a controlled environment (room temperature: $23 \pm 2^\circ\text{C}$, relative humidity: $60 \pm 5\%$, 12 h day/night cycle) with a balanced diet and water *ad libitum*. All the animal experiments were approved by the animal ethical committee, Department of Physiology, Calcutta University, in accordance with the guideline of the committee for the purpose of control and supervision of experiments on animal (CPCSEA Ref No.: 820/04/ac/CPCSEA dated 06.08.2004), Government of India.

2.3. Preparation of S-chitosan

S-chitosan was prepared by ring-opening reaction using succinic anhydride in dimethyl sulfoxide according to the previous report with slight modifications (Zhou & Wang, 2009). Chitosan (2.0 g) was suspended in 40 ml DMSO containing succinic anhydride and the reaction was carried out for 6 h at $\sim 65^\circ\text{C}$. Then, the mixture was filtered and the precipitate was dispersed in ethanol and kept for 1 h at room temperature. The pH of the dispersion was adjusted to 10–12 using 1 M NaOH and filtered. The product was purified by dissolving again in 90 ml of distilled water and reprecipitated by 270 ml acetone. The final precipitate was filtered and

washed with ethanol followed by acetone wash and finally dried under vacuum at 50°C to get the final product.

2.4. Determination of degree of substitution (DS)

The degree of substitution (DS) of S-chitosan was determined by the potentiometric titration method as our previous report (Mukhopadhyay, Sarkar, Chakraborty, et al., 2013; Mukhopadhyay, Sarkar, Soam, & Kundu, 2013). The degree of substitution (DS) is calculated as follows:

$$\text{DS} = \frac{161 \times A}{m_{\text{NSCTS}} - 96 \times A}$$

$$A = V_{\text{NaOH}} \times C_{\text{NaOH}} \quad (1)$$

where V_{NaOH} and C_{NaOH} are the volume and molarity of aqueous NaOH, respectively, and m_{NSCTS} is the mass of S-chitosan. 161 and 96 are the respective molecular weights of glucosamine (repeating unit of chitosan) and carboxymethyl group.

2.5. Preparation of PAA/S-chitosan

To prepare PAA/S-chitosan, 0.5 g S-chitosan was dissolved in 30 ml of distilled water in a two neck round bottom flask, N_2 gas was purged for 30 min. Then, 0.1 g APS dissolved in 5 ml water was added to the S-chitosan solution and the reaction mixture was continuously stirred for 10 min at 60°C temperature. Different amounts of acrylamide solution in water were added to the above solution, followed by the addition of different amounts of methylenbisacrylamide (MBA) solution in water. Then, the reaction mixture was allowed to stir continuously for 1 h at the same temperature under nitrogen atmosphere. The final product was cooled at room temperature and the resulting bulk hydrogel was cut into small pieces and were kept in methanol at room temperature for 24 h. After complete dewatering, the small hydrogel pieces were removed from methanol and dried under vacuum at 50°C . PAA-chitosan was also prepared as control, following the above procedure.

2.6. Characterization of the hydrogel

2.6.1. FT-IR spectroscopy analysis

Fourier transform infrared (FT-IR) analysis was carried out with ATR-FT-IR (Model-Alpha E, Bruker, Germany) spectrometer, scanning from 4000 to 500 cm^{-1} for 42 consecutive scans at room temperature. Chitosan, and its derivatives were mixed separately with potassium bromide (KBr) at a 1:10 weight ratio and KBr pellets were prepared using 10 tons of hydraulic pressure for 10 min at room temperature for measurements.

2.6.2. X-ray diffraction studies

The powder X-ray diffraction patterns of the polymers were examined using a wide angle X-ray diffractometer (Panalytical X-Ray Diffractometer, model – X'pert Powder), employing $\text{Cu K}\alpha$ filtered radiation ($\lambda = 1.54060$). The XRD scan rate was fixed at 1° min^{-1} and the step size was 0.04° . The accelerating voltage and current used were 40 kV and 30 mA, respectively.

2.6.3. Scanning electron microscopy (SEM)

The morphology of the PAA/S-chitosan was observed under a scanning electron microscope (Hitachi, Model: 3400N). The hydrogels were allowed to reach equilibrium swelling and cut into small pieces to expose the inner surface. Then, they were freeze dried using a freeze dryer (Operon, Model: FDU 8606, Korea) to remove the water completely. Then, the freeze-dried cross-sections were exposed to SEM. Prior to imaging the freeze-dried cross-sections

were taped on SEM stub and sputter coated with a thin layer of gold under vacuum to neutralize the charging effects before scanning under SEM at an acceleration voltage of 15 kV.

2.7. Swelling property of the hydrogel

The swelling characteristics of the hydrogels in different pH media, i.e., pH 1.2 (simulated gastric fluid), pH 6.8 (simulated intestinal fluid) prepared following U.S. Pharmacopeia were investigated following our previous report (Mukhopadhyay, Sarkar, Chakraborty, et al., 2013; Mukhopadhyay, Sarkar, Soam, et al., 2013).

The swelling ratio (Q_s) was calculated using the following equation:

$$Q_s = \frac{W_s - W_d}{W_d} \quad (2)$$

where W_s and W_d denote the weight of the swollen beads and the weight of the dry hydrogels, respectively.

2.8. Insulin loading and encapsulation within the hydrogels

The hydrophilic insulin was loaded into the hydrogels using swelling-diffusion method. Insulin solution (1 mg/ml) was prepared in phosphate buffer (pH 7.4). The dried cylindrical hydrogels were placed into 10 ml of insulin solution and allowed to swell for 1 day at 20 °C till equilibrium swelling. The amount of insulin left in the loading medium was determined by UV-vis spectroscopy (OPTIGEN POP BIO, Mecasys Co. Ltd., Korea) following standard Lowry's protein assay at 660 nm. The insulin loading and encapsulation efficiency were determined by the following formula.

$$\text{Insulin loading (\%)} = \frac{\text{Total amount of insulin in hydrogels}}{\text{Total amount of hydrogels}} \times 100 \quad (3)$$

Encapsulation efficiency (%)

$$= \frac{\text{Actual loading of insulin in hydrogels}}{\text{Theoretical loading of insulin in hydrogels}} \times 100 \quad (4)$$

2.9. In vitro insulin release profiles of hydrogels

To evaluate the insulin release profile, hydrogels were immersed in buffer solution at pH corresponding to GI tract (i.e., pH 1.2, pH 6.8 and pH 7.4). At specific time intervals, an aliquot of the sample (200 μ l) was taken and the corresponding concentration of the released insulin was determined by standard Lowry's protein assay using a UV spectrophotometer at 660 nm.

2.10. Mathematical modeling of release kinetics (supplementary material)

To determine the insulin release mechanism, the *in vitro* insulin release data were fitted with standard zero-order, first-order, Higuchi and Korsmeyer–Peppas models (Ritger & Peppas, 1987; Zhang et al., 2009).

2.11. Mucoadhesion study

2.11.1. Mucin adsorption assay of hydrogels

To determine the mucin adsorption capacity of hydrogels, a periodic acid/Schiff (PAS) colorimetric method was performed (Mantle & Allen, 1978) (details attached as supplementary materials).

2.11.2. Ex vivo mucoadhesion studies

Mucoadhesion studies were carried out on freshly excised mice small intestinal tissue according to previously described method with slight modification (Sajeesh et al., 2006).

2.12. In vivo pharmacological response of hydrogels

Diabetes was induced in male Swiss albino mice (25–28 g) according to our previous report (Mukhopadhyay, Sarkar, Chakraborty, et al., 2013; Mukhopadhyay, Sarkar, Soam, et al., 2013). Hydrogels loaded with (50 and 100 IU kg^{−1} b.w./day) dose of insulin were administered orally to the diabetic animals. Mice with subcutaneous injection of insulin solution (5.0 IU kg^{−1} b.w./day) were used as control ($n = 6$ in each group). The blood samples were taken from tail vein and glucose level was checked using Bayer's glucose meter at regular time interval (2 h).

Again, insulin bioavailability at peroral treatment was evaluated. The following hydrogel formulations were administered to diabetic mice individually: group I: oral insulin solution (50 IU kg^{−1} b.w.), group II: oral insulin-loaded PAA/S-chitosan (50 and 100 IU kg^{−1} b.w.), group III: oral insulin-loaded PAA-chitosan (50 IU kg^{−1} b.w.) and group IV: SC injection of insulin solution (5 IU kg^{−1} b.w.). Blood samples were collected from the tail vein and serum was separated by centrifugation at 5000 rpm, for 10 min at 4 °C and stored at −20 °C. Serum insulin concentrations were quantified using enzyme linked immunosorbent assay (ELISA). The relative bioavailability of insulin (Teply et al., 2008) was calculated using the following formula:

$$\text{Relative bioavailability} = \frac{\text{AUC}_{(\text{Oral})} \times \text{DOSE}_{(\text{Sc})}}{\text{AUC}_{(\text{Sc})} \times \text{DOSE}_{(\text{Oral})}} \times 100\% \quad (5)$$

where AUC is the total area under the plasma insulin concentration versus time curve.

2.13. Toxicity assay of the PAA/S-chitosan and PAA-chitosan in vivo models

Acute toxicity studies were carried out with PAA/S-chitosan and PAA-chitosan peroral treatment at a dose of 150 mg b.w./day in Swiss albino mice. Experimental animals were divided into the following 4 groups ($n = 6$). Group I (control): only 0.5 ml 0.9% saline perorally, group II: PAA-chitosan (150 mg kg^{−1} b.w./day) orally, group III: PAA/S-chitosan (150 mg kg^{−1} b.w./day) orally and group IV: PAA/S-chitosan (300 mg kg^{−1} b.w./day) orally.

On the next day, urine was collected and the animals were anesthetized to collect blood from retro-orbital vein. Serum was separated and stored at −20 °C for assessment of different biochemical parameters.

2.13.1. Liver function test

Serum was used to estimate the serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and lactate dehydrogenase activity (LDH) to analyze the liver function hydrogel treated mice.

2.13.2. Nephro-toxicity test

Urine samples were analyzed for quantitative measurement of creatinine (serum and urine) micro-protein to evaluate the nephro toxicity hydrogel treated animals. Again, urine samples were qualitatively analyzed for several biochemical parameters.

For pathohistological diagnosis, liver and kidney were fixed in 10% phosphate buffered formalin, and tissue sections were stained with hematoxylin and eosin (H&E) and observed under microscope.

2.14. Statistical analysis

All the results were expressed as mean $\pm$ SE, $n = 6$. The significance level was determined by one-way ANOVA following Tukey's post hoc test. $p < 0.05$ was considered as significant.

3. Results and discussion

3.1. Characterization of polymers

PAA/S-chitosan is prepared involving graft copolymerization reaction of acrylamide onto S-chitosan backbones in the presence of APS and MBA. Ammonium persulphate produces sulfate anion-radical by thermal decomposition and subtracts the hydrogen from one of the functional groups of chitosan (i.e., COOH, OH, and or NH_2) to form the corresponding radical (macroradical). Then, the

Table 1a

Composition of S-chitosan with degree of substitution value.

Sample name	Composition weight ratio (g/g)	Yield (%)	Degree of substitution (DS)
S-chitosan 1	Chitosan:succinic anhydride (1:1)	81.5	0.58
S-chitosan 2	Chitosan:succinic anhydride (1:2)	89	0.69

resulting macroradicals initiate graft copolymerization with acrylamide, leading to a crosslinked graft copolymer due to the presence of crosslinking agent, i.e., MBA.

The yield (%) and degree of substitution (DS) of S-chitosan are shown in Table 1a. Different formulations of PAA/S-chitosan hydrogels are shown in Table 1b.

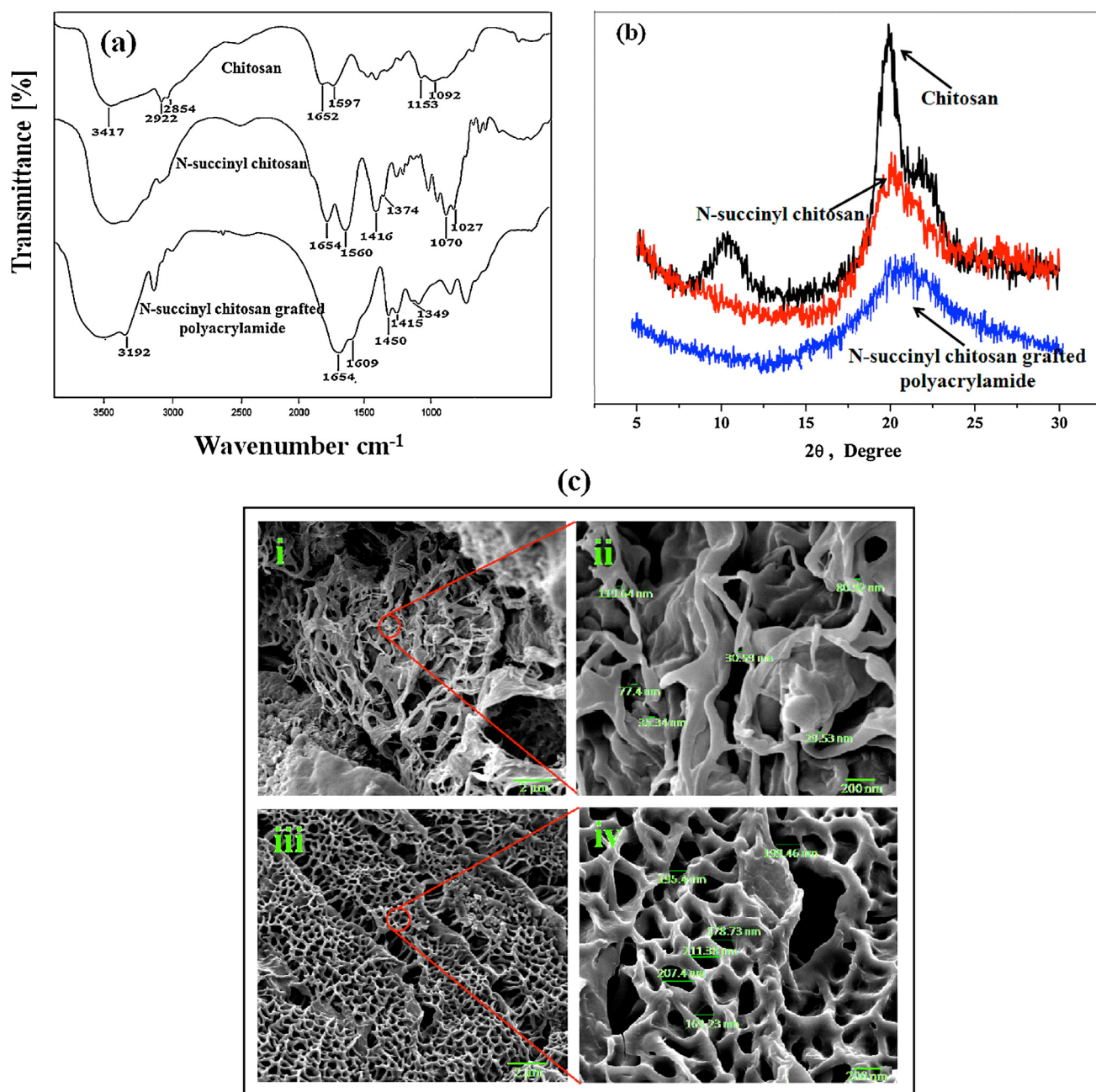


Fig. 1. The FT-IR spectra of chitosan and its derivatives (a), the X-ray diffraction spectrum of chitosan and its derivatives (b), the SEM images of insulin loaded hydrogels (c), crosslinked with MBA 0.1 g (i), magnified view (ii) and crosslinked with MBA 0.05 g (iii), magnified view (iv).

Table 1b

Different composition of prepared hydrogels.

Sample code	Composition weight ratio (g/g)	Amount of MBA (g)
S1	Chitosan:AAM (1:2)	0.1
S2	S-chitosan 1:AAM (1:1)	0.05
S3	S-chitosan 2:AAM (1:2)	0.05
S4	S-chitosan 1:AAM (1:1)	0.1
S5	S-chitosan 2:AAM (1:2)	0.1

The FT-IR spectra of chitosan, S-chitosan, and PAA/S-chitosan are shown in Fig. 1a. The basic characteristic peaks of chitosan were found at 3417 cm^{-1} (O–H stretch and N–H stretch, overlap), 2922 and 2854 cm^{-1} (C–H stretch), 1652 cm^{-1} (NH_2 deformation), 1153 cm^{-1} (bridge-O– stretch) and 1092 cm^{-1} (C–O stretch) (Mukhopadhyay, Sarkar, Chakraborty, et al., 2013; Mukhopadhyay, Sarkar, Soam, et al., 2013). The absorption band of S-chitosan at 1416 cm^{-1} could be attributed to the symmetric stretching of the COO^- , providing direct evidence for the successful modification of chitosan with succinic anhydride. Moreover, the peak at 1597 cm^{-1} (NH_2 bending) greatly weakened and a new signal appeared at 1560 cm^{-1} (assigned to the secondary amines) further indicated

the substitution of the chitosan (Zhou & Wang, 2009). Again, the IR spectrum of PAA/S-chitosan showed few new absorption peaks at 3192 cm^{-1} (NH_2), 1609 cm^{-1} (NH_2), 1450 cm^{-1} (C–H) and 1349 cm^{-1} , indicating the existence of polyacrylamide chain in the prepared hydrogel (Li, Zhang, & Wang, 2007).

The X-ray diffraction of chitosan, S-chitosan and PAA/S-chitosan is shown in Fig. 1b. Chitosan showed two different peaks at $2\theta = 10^\circ$ and $2\theta = 20^\circ$. The peak at 10° was assigned to the crystal form I and the strong peak at 20° was assigned to form II (Fig. 1b). But, for both S-chitosan and PAA/S-chitosan, the peak at 10° disappeared and the reflection at 20° also significantly decreased (Zhou & Wang, 2009). This may happen due to the destruction of intermolecular hydrogen bonds between amine groups and hydroxyl groups of the native chitosan. The results clearly indicated that the graft copolymerization caused destruction of the ordered crystal structure of the native chitosan.

3.2. Scanning electron microscope (SEM) of hydrogel

The SEM micrographs of insulin loaded freeze dried PAA/S-chitosan crosslinked with 0.1 g and 0.05 g MBA are shown in

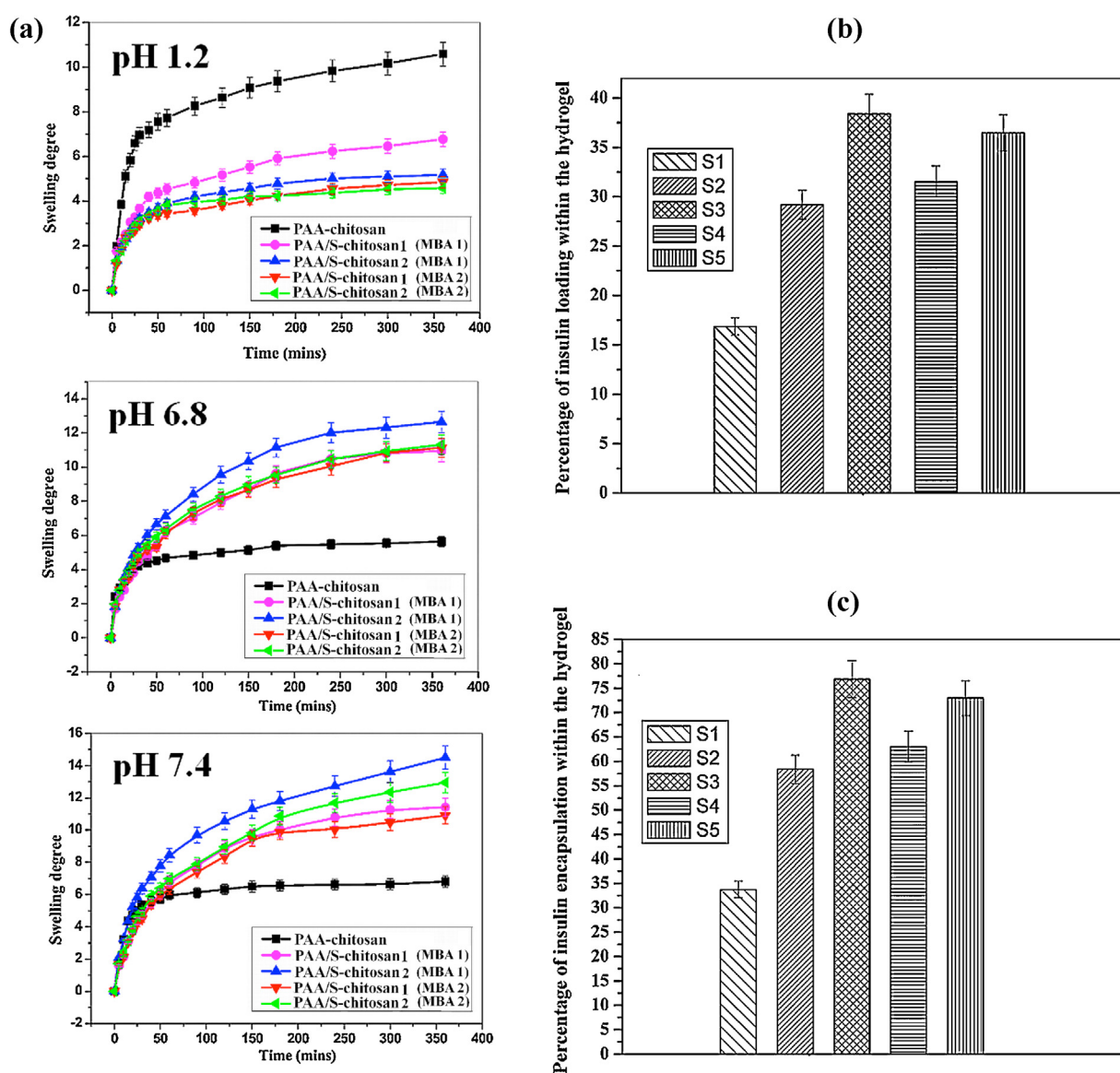


Fig. 2. The swelling ratio of hydrogels in different pH media (a), percentage of insulin loading in the hydrogels (b), insulin encapsulation efficiency of the hydrogels (c).

Fig. 1c (i) and (iii); these micrographs confirmed their highly porous structure. These porous architectures should be beneficial for encapsulation of insulin within the hydrogels. It was also noticed that higher amount of MBA (0.1 g) generated more compact hydrogels with small pores (27–120 nm) [Fig. 1c (ii)] than lower amount of MBA (0.05 g) (shown in Table 1b) crosslinked hydrogels, where pores found to be little larger [150–220 nm; Fig. 1c (iv)]. So, the concentration of crosslinker has an important impact in hydrogel preparation. Probably, the pores arose from the freeze-drying, during which water was evaporated from the hydrogel and pores were left in the networks (Zhang et al., 2010). The porous hydrogels could protect the encapsulated insulin from the protease degradation (Yin, Ding, & Fei, 2008). Porous structure enables their fast swelling, thereby allowing the hydrogels to quickly entrap the enzymes, while chelating as well as adsorption properties of the carboxyl group on *N*-succinylchitosan-graft-poly(acrylamide) and *N*-succinyl chitosan for Ca^{2+} led to deprivation of Ca^{2+} from trypsin and chymotrypsin structures, resulting in successive inactivation of the enzymes, in turn protecting encapsulated insulin. Moreover, increased porosity will facilitate faster diffusion of buffer through

the hydrogel network, in turn facilitating insulin release from the hydrogels.

3.3. Swelling behavior of hydrogels

The swelling study of hydrogels at pH 1.2, 6.8 and 7.4 buffers (Fig. 2a) revealed that PAA/S-chitosan swelled very little in comparison to PAA-chitosan. A significant swelling of PAA-chitosan at pH 1.2 is due to the protonation of primary amino groups ($-\text{NH}_3^+$) on chitosan, creating repulsive force and leading to expansion of the gel network. Whereas, at acidic pH, carboxyl groups ($-\text{COO}^-$) remained protonated (i.e., $-\text{COOH}$), leading to shrinkage of the hydrogel network (Li et al., 2007). Increasing amount of carboxyl groups ($-\text{COO}^-$) on S-chitosan (higher degree of succinylation of chitosan) allows more decreased swelling of PAA/S-chitosan in 1.2 pH (~6 h incubation) as observed in Fig. 2a. With gradual increase in the pH, (from acid to neutral), the swelling behavior of both the PAA-chitosan and PAA/S-chitosan was altered accordingly. At pH 6.8 and 7.4 (intestinal), the PAA/S-chitosan showed controlled swelling for 6 h at 37 °C. All the PAA/S-chitosan started swelling

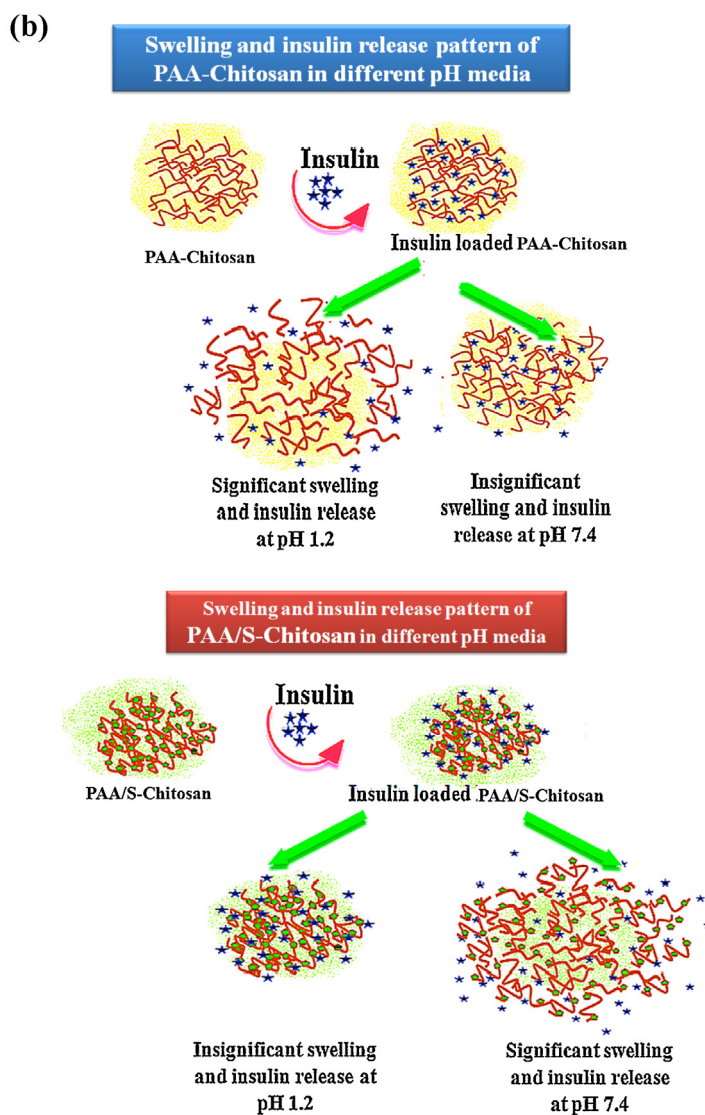
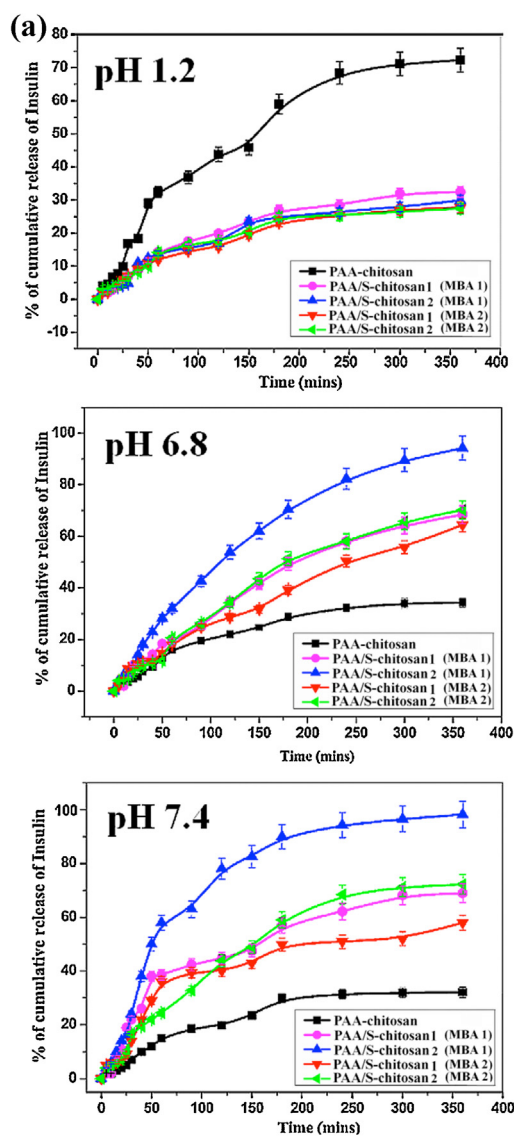


Fig. 3. The *in vitro* insulin release profile of hydrogels (a), schematic presentation of swelling and insulin release pattern of the PAA-chitosan and PAA/S-chitosan at pH 1.2 (stomach) and pH 7.4 (intestine) (b).

after 30 min of incubation and gradually swelled for 6 h till an equilibrium state was reached Fig. 2a. On the other hand, PAA-chitosan showed very little swelling (swelling degree ~ 5) at pH 6.8 and 7.4 for same time of incubation. At higher pH, carboxylic acid groups remain in COO^- form, resulting in repulsive electrostatic force between the ionized groups of COO^- on S-chitosan. This repulsion force between negatively charged COO^- ions made the hydrogels swell in alkaline pH (6.8 and 7.4) in a sustained manner (Li et al., 2007).

In the present work, two different concentrations of MBA (0.05 g and 0.1 g, shown in Table 1b) were used to vary the crosslinking density because it also influences the swelling kinetics of hydrogels. From Fig. 2a, it is observed that PAA/S-chitosan crosslinked with less amount of MBA (0.05 g) showed more gradual swelling over a long period in comparison to higher MBA (0.1 g) crosslinked hydrogels.

Therefore, it is observed that PAA/S-chitosan will be more fruitful than PAA-chitosan in releasing insulin in the intestinal environment (pH 6.8, 7.4) and protecting it from the harsh acidic stomach (pH 1.2).

3.4. Insulin loading of hydrogels

It was observed that PAA/S-chitosan showed better insulin loading Fig. 2b and insulin encapsulation efficiency (Fig. 2c) as compared to that of PAA-chitosan. It was found that PAA/S-chitosan (S3)

showed the maximum insulin loading and encapsulation efficiency of 46% and 76%, respectively. But, PAA-chitosan (S1) showed poor insulin loading (22%) as well as encapsulation efficiency (33%). The increased acrylamide concentration might improve insulin loading, providing electrostatic interaction between insulin and amino groups of acrylamide in the hydrogels. But higher monomer concentration might result in preferential homo-polymerization over graft co-polymerization, in turn affecting the viscosity of the reaction medium hindering the movement of free radicals and monomer molecules. Again, decreased crosslinker concentration would result in significant swelling, enabling better insulin loading.

3.5. In vitro insulin release profile of hydrogels

In vitro insulin release study is shown in Fig. 3a. At pH 1.2, only 20–25% of encapsulated insulin was released from all the PAA/S-chitosan, whereas 73% insulin was released from PAA-chitosan (Fig. 3a). Because in acidic pH 1.2, the protonation of carboxyl groups (COOH) on S-chitosan probably limits the insulin release with progressive shrinkage of the PAA/S-chitosan. On the contrary, PAA-chitosan released higher amount of insulin ($\sim 73\%$) as it swelled due to protonation of free primary amino groups (NH_3^+) on chitosan structure creating repulsive force at the same pH 1.2.

An increased amount of insulin was released from PAA/S-chitosan in a sustained way in the simulated fluid of intestine (pH 6.8 and 7.4). Almost all of the different formulations of

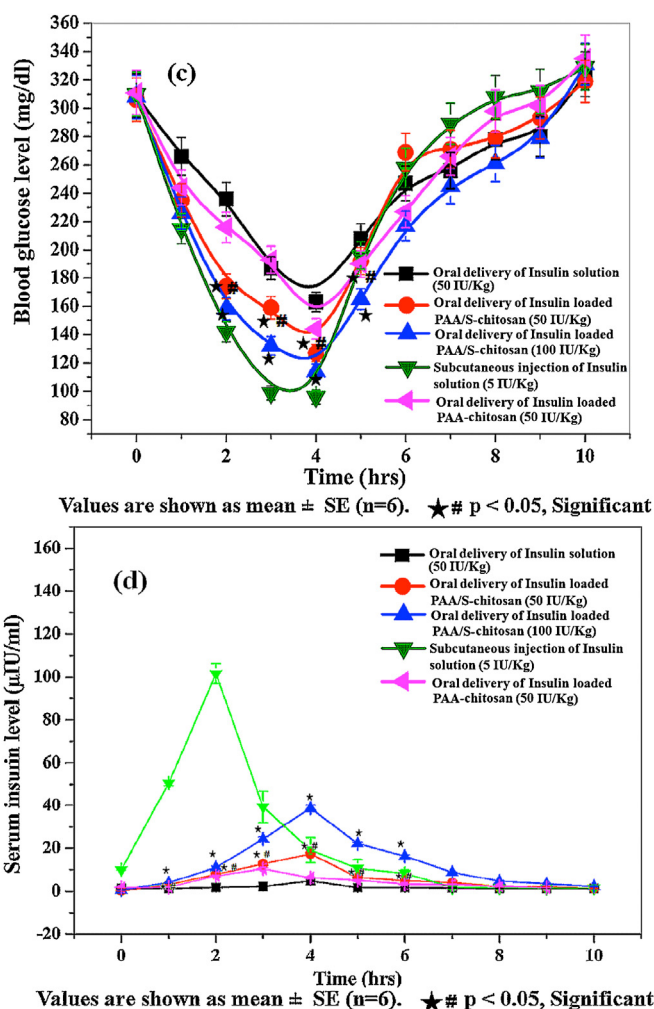
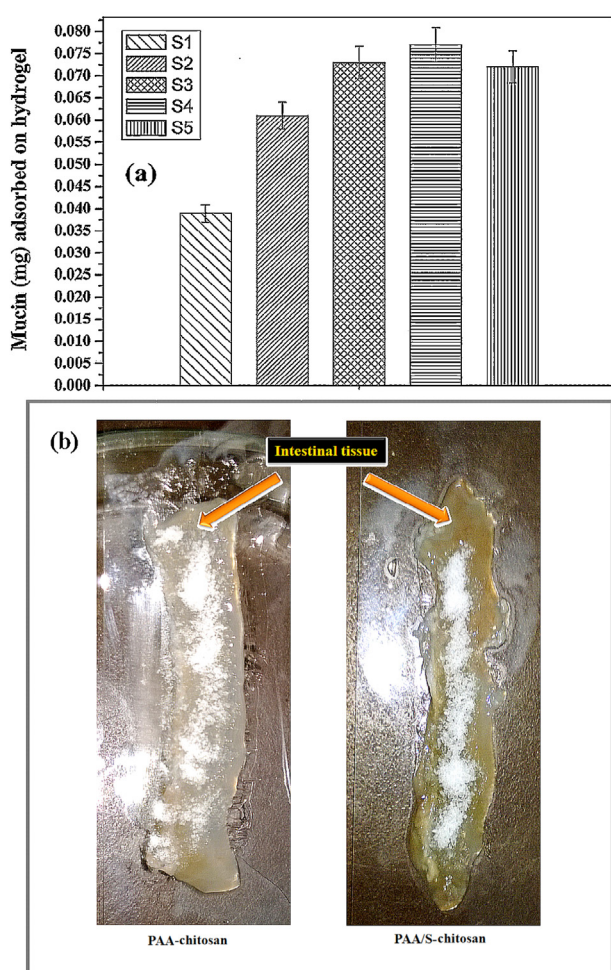


Fig. 4. Mucin adsorption study of hydrogels (a), mucoadhesive property of the hydrogels in mouse intestinal tissue (b) (pictures taken after 30 min washing with phosphate buffer, pH 7.4). In vivo hypoglycemic effect of insulin loaded hydrogels (c), concentration of serum insulin after peroral administration of insulin loaded hydrogels (d).

PAA/S-chitosan started swelling slowly after 30 min of incubation at pH 6.8 and pH 7.4 (intestine) and almost entire entrapped insulin (~98%) was released because of the high electrostatic repulsion force between the ionized carboxylic groups on polymer structure (Fig. 3a). So, both at the pH of duodenum and ileum, insulin was released in a controlled manner over a period of 6 h. On the other hand, PAA-chitosan showed only 20–26% of insulin release at pH 6.8 and pH 7.4. Mura et al. (2011) also showed pH sensitive swelling of S-chitosan matrix in colonic drug delivery. Thus, PAA/S-chitosan found to be more effective in releasing insulin in a sustained fashion in intestine, while protecting it from harsh stomach environment. Fig. 3b shows the comparative swelling and release of insulin from PAA-chitosan and PAA/S-chitosan in a pH gradient buffer. To deduce the insulin release mechanism, the parameters 'n' and 'k' of the Korsmeyer–Peppas equation were computed, that showed an anomalous non-Fickian diffusion mechanism from the hydrogel matrices.

3.6. Assessment of the mucoadhesive behavior of hydrogels

3.6.1. Mucus glycoprotein assay

It is noticed from Fig. 4a, that the PAA-chitosan adsorbs lower amount of mucin in comparison to the different formulations

of PAA/S-chitosan. Only 39% of mucin is adsorbed in case of PAA-chitosan, where as of 77% mucin absorption is noticed for PAA/S-chitosan (S3).

3.6.2. Mucoadhesion studies in animal model

Fig. 4b depicts that PAA/S-chitosan have better mucoadhesive property compared to PAA-chitosan on mouse intestine even after continuous washing with buffer (pH 7.4) supporting the *in vitro* mucin adsorption study.

3.7. In vivo pharmacological response of insulin loaded PAA/S-chitosan, serum insulin concentration and insulin bioavailability

In vivo responses of insulin loaded PAA/S-chitosan hydrogels are illustrated in Fig. 4c. The insulin loaded PAA/S-chitosan showed better hypoglycemic effects in a dose dependent manner (50 IU and 100 IU kg⁻¹ b.w.) in comparison to insulin loaded PAA-chitosan. Generally, subcutaneous administration of the insulin (5 IU kg⁻¹ b.w.) shows hypoglycemic effects within 30–45 min and blood sugar level reaches (96 mg/dl) at 3 h after injection. Subsequently, an increase in blood glucose level was also noticed with time and returned to glycemic level again (Fig. 4c). Orally administrated insulin (50 IU kg⁻¹ b.w.) was not able to reduce

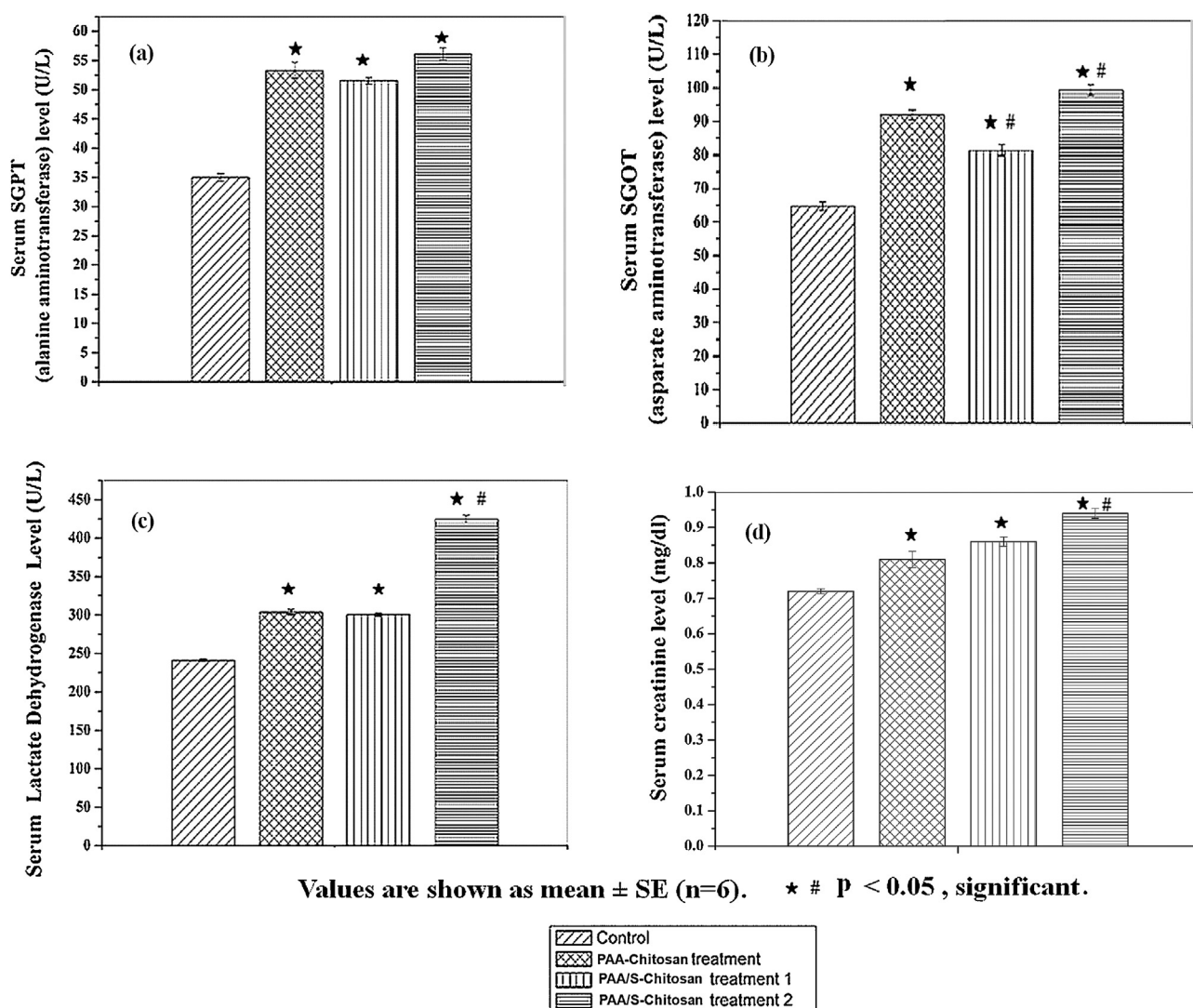


Fig. 5. Acute hepato-toxicity study of the hydrogel treated animals. Serum SGPT (a), serum SGOT (b), serum LDH (c), serum creatinine (d).

glycemia (163 mg/dl) significantly in diabetic mice due to enzymatic degradation in the GI tract. From Fig. 4c, it is observed that insulin ($50 \text{ IU kg}^{-1} \text{ b.w.}$) loaded PAA/S-chitosan reduced blood glucose level up to 127 mg/dl, and insulin loaded PAA-chitosan ($50 \text{ IU kg}^{-1} \text{ b.w.}$) reduced up to 144 mg/dl. Again, insulin loaded PAA/S-chitosan at higher dose ($100 \text{ IU kg}^{-1} \text{ b.w.}$) reduced blood sugar level 114 mg/dl after 2 h of oral administration and sustained at least up to 6 h. So, PAA/S-chitosan was able to protect insulin from degradation reducing glycemia in diabetic model at peroral delivery.

The corresponding serum insulin concentrations at different time profiles were studied in mice to evaluate the relative insulin bioavailability (Fig. 4d). The animal group with subcutaneously injected insulin, resulted in a maximum serum insulin concentration at 2 h post injection, whereas insulin loaded PAA/S-chitosan at a dose of $100 \text{ IU kg}^{-1} \text{ b.w.}$ showed a maximum concentration of serum insulin at 4 h of administration. In contrast, negligible amount of serum insulin was detected with only peroral insulin. From the areas under the curves ($\text{AUC}_{(0-10 \text{ h})}$) of the group in which insulin loaded PAA/S-chitosan was orally administered, the relative bioavailability of insulin was found to be $\sim 4.43\%$. These results clearly illustrated that the intestinal absorption of insulin was significantly enhanced using the PAA/S-chitosan after oral delivery. Nakamura et al. (2004) reported an in situ closed-loop method to investigate the

pharmacological efficacy of insulin-loaded polymer (ILP) to rat intestinal segments, producing 4.6% pharmaceutical bioavailability at a dose of 25 IU kg^{-1} . Again, Lowman et al. (1999) reported bioavailability of $\sim 2.44\%$, in diabetic rat using poly(methacrylic-g-ethylene glycol) hydrogels at a dose of 25 IU kg^{-1} body weight.

3.8. Evaluation of in vivo toxicity after oral administration of hydrogels

3.8.1. Liver function test analysis

The liver specific enzyme ALAT (alanine aminotransferase) and ASAT (aspartate aminotransferase) are significantly elevated in hepatobiliary diseases with a direct correlation with damages of liver parenchyma. It is noticed that the ALAT value (Fig. 5a) of control animal is 35.02 U/L, PAA-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 53.3 U/L and PAA/S-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 51.56 U/L and 56.13 U/L ($300 \text{ mg kg}^{-1} \text{ b.w.}$). The reference range is 28–184 U/L in mouse (Olfert, Cross, & McWilliam, 1993). Similarly, the ASAT value of control animal is 64.59 U/L, PAA-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 90.74 U/L and PAA/S-chitosan treated animal is 80.33 U/L ($150 \text{ mg kg}^{-1} \text{ b.w.}$) and 99.79 U/L ($300 \text{ mg kg}^{-1} \text{ b.w.}$) as shown in Fig. 5b. The reference range of SGOT is 55–251 U/L. Again, it is observed from Fig. 5c, that the LDH value of control animal is 241.22 U/L,

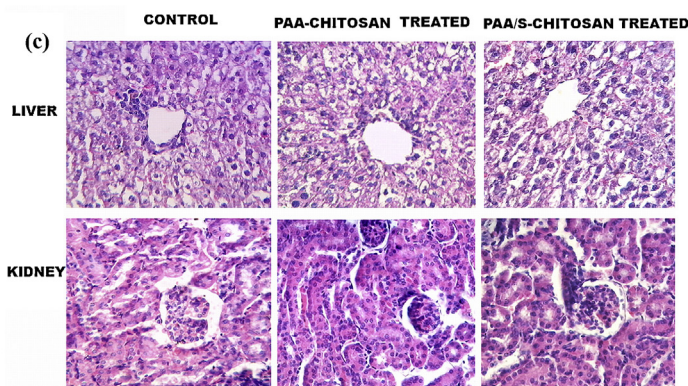
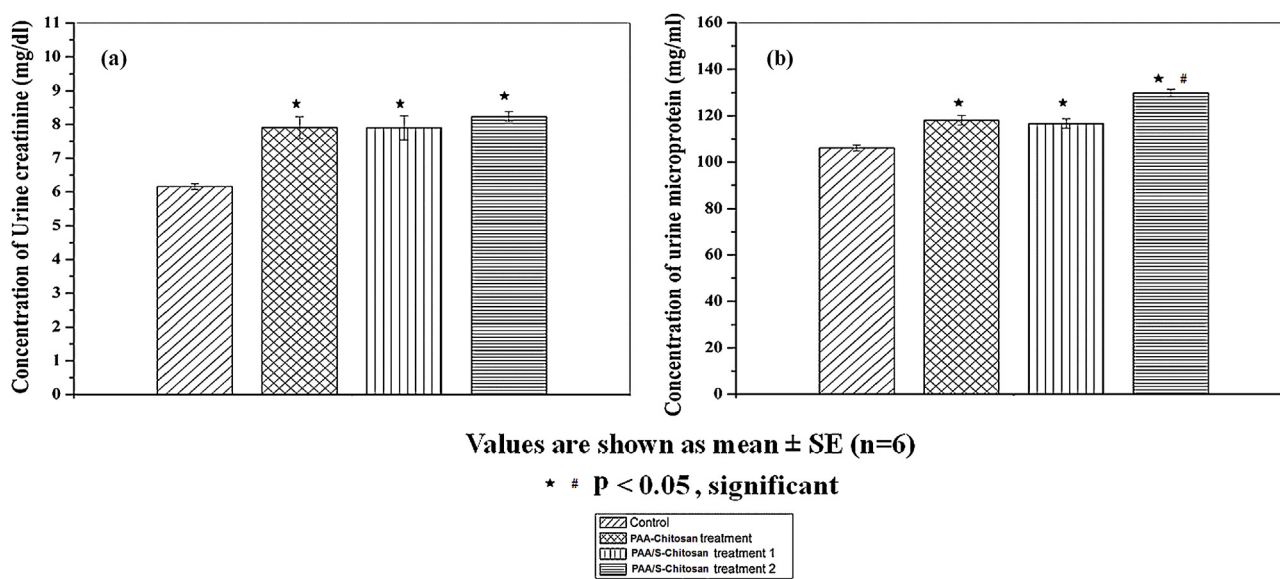


Fig. 6. Acute nephro-toxicity study of the hydrogel treated animals. Concentration of urine creatinine (a), urine microprotein (b), pathohistological observation of liver and kidney sections (magnification $40\times$) (c).

Table 2
Qualitative analysis of different biochemical parameters after peroral treatment of hydrogels.

Parameters	Control animal	Treatment PAA-chitosan (150 mg)	Treatment PAA/S-chitosan (150 mg)	Treatment PAA/S-chitosan (300 mg)
Urobilinogen	1	1	0.2	2
Protein	Negligible	Negligible	Negligible	Trace
pH	7.5	7.5	7.0	7.5
Blood	Moderate (non-hemolyzed)	Moderate (hemolyzed)	Moderate (non-hemolyzed)	Trace (hemolyzed)
Specific gravity	1.020	1.015	1.010	1.025
Ketone	15–30	40	15–30	40–80
Bilirubin	Negligible	+	Negligible	++
Glucose	Negligible	Negligible	Negligible	Negligible

PAA-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 303.88 U/L and PAA/S-chitosan treated animal is 300.49 U/L ($150 \text{ mg kg}^{-1} \text{ b.w.}$) and 424.65 U/L ($300 \text{ mg kg}^{-1} \text{ b.w.}$) are within the LDH reference range of $230\text{--}460 \text{ U/L}$. Although significant changes in SGPT, SGOT and LDH value is found in comparison to control, but all values come under reference range, suggesting no liver damage, toxicity and functional disorder due to peroral treatment of PAA/S-chitosan.

3.8.2. Assessment of nephro-toxicity

The serum creatinine of control animal is 0.72 mg/dl , PAA-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 0.81 mg/dl and PAA/S-chitosan treated animal is 0.86 mg/dl ($150 \text{ mg kg}^{-1} \text{ b.w.}$) and 0.94 mg/dl ($300 \text{ mg kg}^{-1} \text{ b.w.}$) as shown in Fig. 5d. Significant change is observed as compared to control group but the values fall within normal reference range of $0.7\text{--}1.1 \text{ mg/dl}$. Significant changes in urine creatinine (Fig. 6a) were also obtained in treated groups in comparison to control but, values are within the reference range ($8.4\text{--}24.6 \text{ mg kg}^{-1} \text{ b.w.}$), indicating no nephro-toxicity. Proteins (albumin and globulin fractions) are known to involve in the maintenance of normal distribution of water between blood and the tissues. Proteinuria may occur mainly due to increased glomerular permeability or defective tubular reabsorption. Fig. 6b shows the concentration of urine microprotein in control animal is 106.12 mg/24 h , PAA-chitosan ($150 \text{ mg kg}^{-1} \text{ b.w.}$) treated animal is 118.05 mg/24 h and PAA/S-chitosan treated animal is 116.6 mg/24 h ($150 \text{ mg kg}^{-1} \text{ b.w.}$) and 129.88 mg/24 h ($300 \text{ mg kg}^{-1} \text{ b.w.}$). A significant change is observed as compared to the control group but all the values are within normal microprotein reference range of $28\text{--}140 \text{ mg/24 h}$ indicating no renal toxicity after peroral delivery of PAA/S-chitosan.

Again in pathological study (Fig. 6c) overall, the appearance of the PAA/S-chitosan treated and PAA-chitosan treated liver sections were similar to the control tissue. The central vein with radiating intact hepatic cells was found in control and treated sections indicating no apparent hepatic toxicity of the hydrogel. Furthermore, the sections of the kidney showed the presence of the renal corpuscle and kidney tubules lined by simple cuboidal epithelium. The renal corpuscle is surrounded by Bowman's capsule. A urinary space (appears as a clear space) is visible on histological slides. The glomerulus, a tuft of capillaries, appears as a large cellular mass. These observations imply no renal toxicity after oral treatment of the hydrogels.

Moreover, qualitative analysis of different biochemical parameters (Table 2), show no significant change after hydrogel treatment.

4. Conclusions

Overall, the results indicated successful preparation of a pH sensitive, porous PAA/S-chitosan and its application in oral insulin delivery. Indeed, the PAA/S-chitosan was able produce better controlled release of insulin following a non-fickian mechanism, which was quite low in acidic stomach and almost complete in alkaline medium, as compared to the native PAA-chitosan. Finally, insulin loaded PAA/S-chitosan showed significant hypoglycemic effects in

diabetic mice after delivery, producing insulin relative bioavailability of $\sim 4.43\%$. Moreover, *in vivo* toxicity and histopathological study demonstrated no toxicity (liver, kidney) at peroral treatment of the hydrogels. Hence, this PAA/S-chitosan seems to be a promising candidate for successful oral insulin administration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2014.06.045>.

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