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Assessment of *in vivo* chronic toxicity of chitosan and its derivates used as oral insulin carriers

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Considering public health protection, the carrier system for oral insulin must be safe. Hence, in the present study, the chronic oral toxicity of chitosan derivates was investigated in a mouse model. Oral administration of polymers did not cause any significant change in the behavioural pattern, body weight, and clinical symptoms of the treated mice. There were also no significant alterations in the biochemical parameters of blood serum and urine. Further, histopathological examination revealed an almost normal architecture, suggesting no significant adverse effects on the liver, kidney and intestine of the treated animals. An *in vitro* haemolysis assay proved that chitosan and its derivatives were blood compatible. Finally, intestinal luminal bacteria were able to biodegrade the polymers completely. Overall, the results suggested that the oral administration of the derivatives of chitosan in mice did not produce any significant toxicity in chronic treatment. Hence, these polymers could be utilized as safe devices for oral delivery of insulin and also other drugs.

1. Introduction

Over the past several decades, enormous progress in the field of biotechnology has led to different bioengineered products for biomedical applications. Among these, polymeric hydrogels, microparticles and nanoparticles have drawn appreciable attention from the pharmaceutical scientists in drug delivery research due to their versatility in targeting tissues, enabling deep molecular targets as well as sustained drug release. Apart from drug delivery, polymeric formulations also displayed excellent efficiency as carriers of potent proteins, DNA, peptides and vaccines. Again, polymers (natural and synthetic) successfully favour versatile routes of administration of several bio-macromolecules (insulin, DNA, other proteins).[1] Diabetes being pandemic and a predicted doubling in diabetes-related deaths between 2005 and 2030,[2] means that the use of polymers in diabetic research has become a popular topic. To date, insulin is the most effective treatment of diabetes and research has focused on alternative routes of insulin delivery using different bioengineered materials[3] to overcome the poor patient compliance with parenteral insulin produced by needle phobia, pain, allergic reactions, skin bulges, common infections, and stress generated from the difficult and long term regimen of insulin therapy.[4,5] However, the oral delivery of insulin, unlike many other therapeutic strategies, remains unsuccessful due to different physicochemical barriers like rapid enzymatic degradation, poor intestinal absorption and poor bioabsorption due to the tight junction between intestinal cells.[6] Natural polymers like agar, agarose, alginate, pectin, gelatine, chitosan, *etc.*, are comparatively more preferred over synthetic polymers with respect to their biodegradable, biocompatible, low-toxic, non-immunogenic and mucoadhesive properties.

Chitosan (derived from chitin by alkaline hydrolysis), one of the most popular biopolymers exhibiting several essential features like biocompatibility, biodegradability, non-toxicity and non-immunogenicity, has been extensively studied in oral insulin research.[7] Chitosan is a linear and partly acetylated (1→4)-2-amino-2-deoxy-β-D-glucan[8,9] that offers certain advantages over other natural polymers in formulating hydrogels, microparticles, and nanoparticles for oral administration of insulin.[10–12] This mucoadhesive, cationic chitosan (due to protonation of the amine groups in acidic pH) not only facilitates effective encapsulation of biomolecules like proteins, drugs, but also prolongs the residence time of insulin in the gastrointestinal tract[13] with impressive enhancement of permeation. However, the poor water solubility of native chitosan[11] limits its wide application in the successful oral delivery of insulin. A variety of water soluble chitosan derivatives has been synthesized and reported in this regard, such as carboxymethyl chitosan,[11,14] succinyl chitosan,[15] succinyl chitosan hydrogel,[10] PEGylated chitosan,[16] quaternized chitosan,[17] trimethylated chitosan,[18] PAMAM grafted chitosan,[19,20] *etc.* Although chitosan is reported

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to be non-toxic, the *in vivo* toxicity assay demonstrated that the LD50 of chitosan is 16 g kg^{-1} in mice. Assessment of the toxicity of these modified polymers after peroral treatment is essential as they are used as insulin carriers for animals. Acute toxicity studies of these chitosan derivates have been carried out previously with an *in vivo* model to conclude safety measures.[10,21] However, diabetic patients demand a long-term regimen of insulin therapy, so chronic toxicity must be evaluated in detail.

In the present investigation, we have carried out a chronic toxicity study of modified chitosan molecules used in oral insulin delivery in order to get an idea about the safety of the products as shown in Fig. 1. It is reported that oral insulin is absorbed from the intestinal lumen and is generally transported *via* the hepato-portal circulation,[22] for which reason we have tested the functional properties of liver and kidney. A pathohistological analysis was also conducted to visualize the structural stability of the respective tissues after oral administration of the polymeric products. A haemolytic assay was performed to test the blood compatibility of the modified chitosan molecules after peroral treatment with the chitosan derivates. Finally, a biodegradation study was conducted with intestinal luminal bacteria to establish the fact that all chitosan derivates that are used as a device for oral insulin delivery, are naturally degradable within the animal system without being accumulated and generating no toxicity in the body.

2. Materials and methods

2.1. Materials

Chitosan (MW 365 kDa, degree of deacetylation (DDA) 86%) was obtained from Himedia, India. Low molecular weight

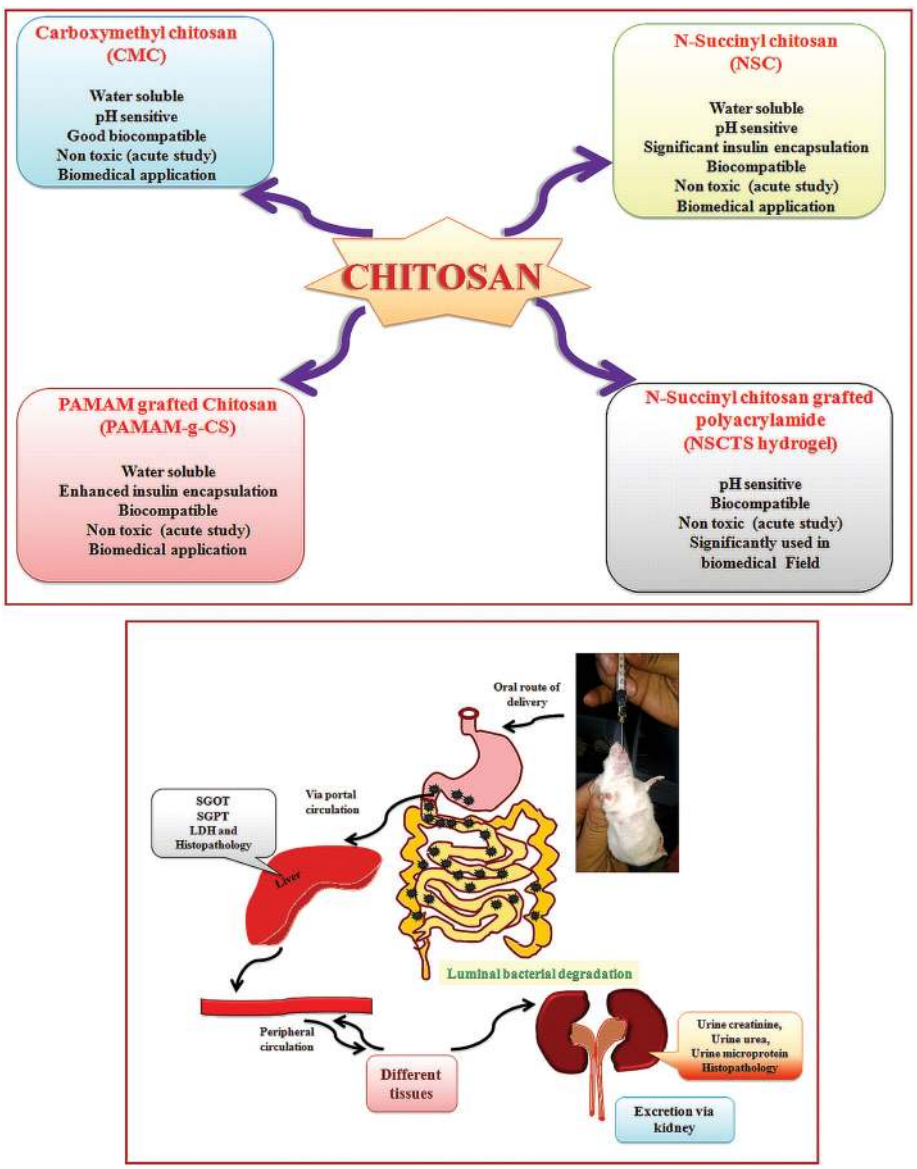

Fig. 1 Chronic toxicity study with modified chitosan materials used as oral insulin carriers.

chitosan (65 kDa, DDA 82%) was prepared using high molecular weight chitosan in the process of oxidative degradation with sodium nitrite (Merck, India) at room temperature according to our previous investigation.[12] A low molecular weight chitosan (65 kDa, DDA 82%) has been used in the study. The molecular weight and degree of deacetylation (DDA) of chitosan were determined using gel permeation chromatography (Waters RI detector, PC2 separation module, using empower 2 software against PEG standard (sigma) calibration curve) and potentiometric titration method.[23,24] Monochloroacetic acid, isopropanol, succinic anhydride, dimethyl sulphoxide (DMSO), ammonium persulfate (APS) were purchased from Sisco Research Laboratories (SRL), India. Acrylamide (AA), methyl acrylate (MA), methylenebisacrylamide (MBA), haematoxylin, eosin, paraffin wax and a creatinine merckotest kit were obtained from Merck (India). A serum glutamate pyruvate transaminase (SGPT) ALAT(GPT)-LS kit and serum glutamate oxaloacetate transaminase (SGOT) AST(GOT)-LS kit were purchased from Piramal Health care Limited, Mumbai, India. A lactate dehydrogenase LDH (P-L) kit and microprotein kit were obtained from Crest biosystems, Goa, India. Multistix reagent strips were used for urine biochemical parameter analysis. All other reagents were of analytical grade and were used directly without further modification.

The carboxymethyl chitosan (CMC) was prepared following the previous report[14] with slight modifications. The *N*-succinyl chitosan (NSC) was prepared as per previous report[15] and *N*-succinyl chitosan grafted polyacrylamide hydrogel (NSCTS hydrogel) was prepared as per our previous study.[10] The PAMAM grafted chitosan (PAMAM-*g*-CS) was prepared as per our previous investigation.[25]

2.2. Animals

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

2.3. Haemolytic assay

An *in vitro* haemolytic assay is considered to be a simple as well as reliable way of measuring blood compatibility of the prepared materials for safe biomedical applications.[24] Furthermore, *in vivo* applications of these polymeric biomaterials will be desirable after investigating the percentage of *in vitro* haemolysis. In this study, we have compared the percentage of haemolysis for different chitosan derivates used for oral insulin delivery in our previous studies.[10–12] Polymeric samples were dissolved in isotonic phosphate buffer solution (pH 7.4). A set of ten centrifuge tubes was arranged containing respectively 5 ml graded concentrations of different polymeric (0.1, 0.25, 0.5, 1, 5, 10, 20 and mg ml^{-1}) in the first eight tubes and the remaining two containing only isotonic phosphate buffer solution (pH 7.4). A haemoglobin suspension (0.5 ml) was added to each of the tubes and mixed gently. Then, the tubes with the polymer samples were incubated at 54 °C for 20 min in a regulated water bath. One of the two tubes containing haemoglobin suspension in isotonic phosphate buffer (pH 7.4) (without polymer) was incubated at 54 °C for 20 minutes and considered as heat control. Whereas the other tube was kept at room temperature and considered to be the normal control. Afterwards, all tubes were centrifuged at 2000 rpm for 3 min and the absorbance (optical density) of the supernatants was recorded at 540 nm using a UV-vis spectrophotometer (OPTIGEN POP BIO, Mecasys Co. Ltd., Korea). The percentage of haemolysis was calculated using the following formula:

$$\%\ \text{of Haemolysis} = \frac{\text{OD3} - \text{OD1}}{\text{OD2} - \text{OD1}} \quad (1)$$

where OD1 = absorbance of normal control (unheated haemoglobin suspension in PBS), OD2 = absorbance of heat control (heated haemoglobin suspension in PBS) and OD3 = absorbance of heat-treated test samples (heated polymeric solutions in PBS with haemoglobin suspension).

2.4. Chronic toxicity assay of chitosan and its derivates in *in vivo* models

Chronic toxicity studies were carried out with chitosan and modified chitosan materials after peroral treatment at a dose of 30 mg kg^{-1} bw per day in Swiss albino mice. Experimental animals were divided into the following seven groups (n = 6). Group I (control): only 0.5 ml of 0.9% saline perorally, group II: chitosan (CS) (30 mg kg^{-1} bw per day) orally, group III: carboxymethyl chitosan (CMC) (30 mg kg^{-1} bw per day) orally, group IV: *N*-succinyl chitosan (NSC) (30 mg kg^{-1} bw per day) orally, group V: *N*-succinyl chitosan grafted polyacrylamide hydrogel (NSCTS hydrogel) (30 mg kg^{-1} bw per day) orally and group VI: PAMAM grafted chitosan (PAMAM-*g*-CS) (30 mg kg^{-1} bw per day) orally, group VII: polyethylenimine (PEI) orally (30 mg kg^{-1} bw per day) as toxic control. The body weight and several behavioural parameters were grossly observed at 7 day intervals in all mice groups under the chronic study. The urine samples were collected after 30 days and the animals were anesthetized to collect blood from the retro-orbital vein. The sera were separated and stored at −20 °C for the assessment of different biochemical parameters.

2.4.1. 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 analyse the liver function of the treated mice.

2.4.2. Nephrotoxicity test. Urine samples were analyzed for quantitative measurement of creatinine (serum and urine), microprotein and urea in urine to evaluate the nephrotoxicity of the biomaterial-treated animals. Again, urine samples were qualitatively analyzed for several biochemical parameters using the strip test.

2.4.3. Pathohistological analysis. After treatment, the animals were dissected to observe any damage that occurred to the internal organs. For the pathohistological diagnosis, liver, kidney and intestine were fixed in 10% phosphate buffered formalin, and tissue sections were stained with haematoxylin and eosin (H&E) and observed under the microscope.

2.5. Biodegradation study of chitosan and its derivates

After successful delivery of insulin *via* the oral route, the polymeric carrier has to be biologically degraded in order to ensure safety. The large intestinal luminal content of the mice was collected to study the effect of luminal bacteria on the biodegradation of the polymers, which have been prepared as potential oral carriers of insulin. The content was serially diluted and the mixed microbial population was traced using the agar plate streaking technique. They were grown together as a mixed microbial consortium in nutrient broth at 37 °C for 24 h. The logarithmic phase culture was aseptically transferred to sterilized vials and the crosslinked polymeric samples namely CS, CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS were carefully dipped into the cultures to ensure a contamination free transfer. Control experiments were also performed in parallel for each polymer with a medium, free of bacterial culture.

All samples were incubated at 37 °C and weight measurements for each polymer were done at 24 h intervals. Aseptic conditions were maintained during the entire process. Scanning electron microscopy (SEM) was also conducted in order to observe the biofilm formation during the biodegradation process.

2.6. Statistical analysis

All results were expressed as mean ± 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. Haemolytic assay

In vitro evaluation of the blood compatibility of the modified chitosan molecules for oral insulin delivery is a prerequisite for further *in vivo* applications. The haemolytic assay is considered to be a simple and reliable way to measure the blood compatibility of prepared materials. In this study, we compared the percentage of haemolysis of the chitosan derivates such as CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS with varying concentrations ranging from 0.1–30 mg ml^{-1} with native chitosan and PEI (25 kDa) which was used as a positive control. All these modified chitosan samples were used as oral insulin carriers in our previous investigations.[10–12] Fig. 2 demonstrates the percentage of haemolysis. It was noticed that slight haemolysis occurred in all cases of polymeric samples after 20 min of incubation at 54 °C, although the percentage of haemolysis is negligible in comparison to that of PEI (25 kDa). Native chitosan and its derivates showed negligible heat-induced haemolysis (ranging from 2–5%), whereas PEI showed a significant haemolysis percentage for each concentration. So, it can be predicted that all chitosan derivates are safe and blood compatible.

3.2. Liver function test

Oral insulin mimics the physiological fate of insulin within the body and diabetic patients need long-term treatment to get completely cured. During the passage through the oral route, major organs like liver and kidney can be affected by the polymers. So, the assessment of chronic toxicity after polymeric treatment must be evaluated in order to check the structural and functional stability of the organs. The liver is one of the most vital organs of the digestive system in vertebrates that plays a pivotal role in metabolism and has a number of functions in the body including glycogen storage, decomposition

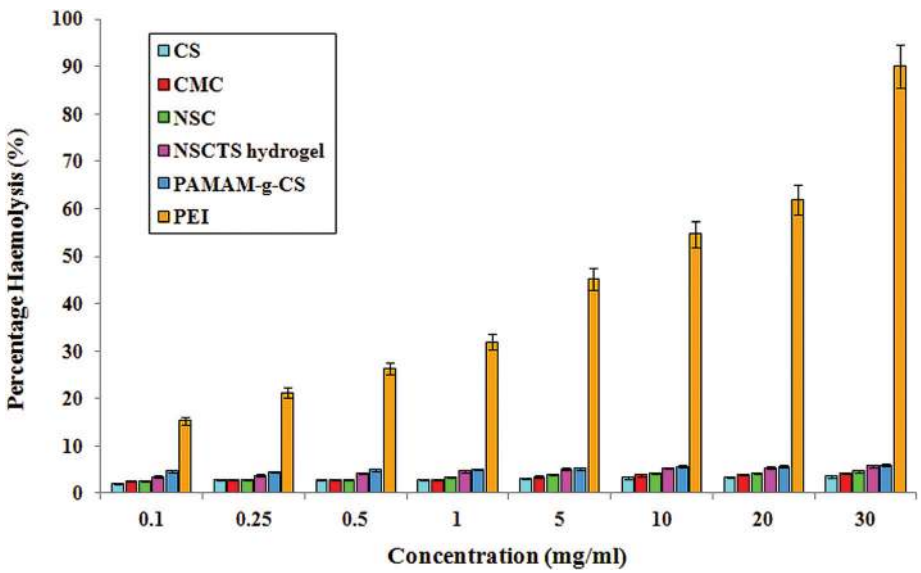

Fig. 2 *In vitro* haemolysis assay of different polymers at the concentration range 0.1–30 mg ml^{-1}.

of red blood cells, plasma protein synthesis, hormone production, and detoxification, protein synthesis, and the production of different biochemicals necessary for digestion. The liver specific enzyme ALAT (alanine aminotransferase) and ASAT (aspartate aminotransferase) are significantly elevated in hepatobiliary diseases with a direct correlation to the damage to liver parenchyma. It is noticed that the SGOT value (Fig. 3a) of the control animal is 69.86 U L^{-1}, that for the CS treated animal is 94.54 U L^{-1}, and for the CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS treated animals the values ranged within 120–152 U L^{-1}, but in the case of the PEI treated animal the SGOT value is 268.54 U L^{-1} at the same dose. The reference range is 55–251 U L^{-1} in mice and all the values fall within that range except for PEI (toxic polymer). So, it can be interpreted that the chitosan derivates do not cause liver toxicity but PEI does. Similarly, the SGPT value is 34.7 U L^{-1} for the control animal, 55.25 U L^{-1} for the CS treated animal, and for the CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS treated animals the SGPT values varied within 79–112 U L^{-1} (Fig. 3b), but in the case of the PEI treated animal the SGPT value increased up to 184.38 U L^{-1} at the same dose. The reference range is 28–184 U L^{-1} in mice. So, it can be said that the chitosan derivates do not affect the liver function and their application is safe as compared to PEI. Again, it is observed from Fig. 3c that the LDH value of the control animal is 251.67 U L^{-1}, and for the CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS treated animals the values varied between 288–409 U L^{-1}; these values are within the LDH reference range of 230–460 U L^{-1}. The LDH level is only found to be elevated in the PEI treatment. Although significant changes in the SGPT, SGOT and LDH values are found in comparison to the control one, all values are within the reference range, suggesting no liver damage, toxicity or functional disorder due to the peroral treatment with modified chitosan molecules in animals. PEI is an efficient carrier of bio-macromolecules but the results indicate that it is toxic during chronic treatment. Therefore, chitosan and its water soluble derivatives serve as non-toxic devices for the oral insulin long-term therapy.

3.3. Assessment of nephrotoxicity

In vertebrates, kidneys serve several essential regulatory functions in the body. Generally, excess organic molecules and

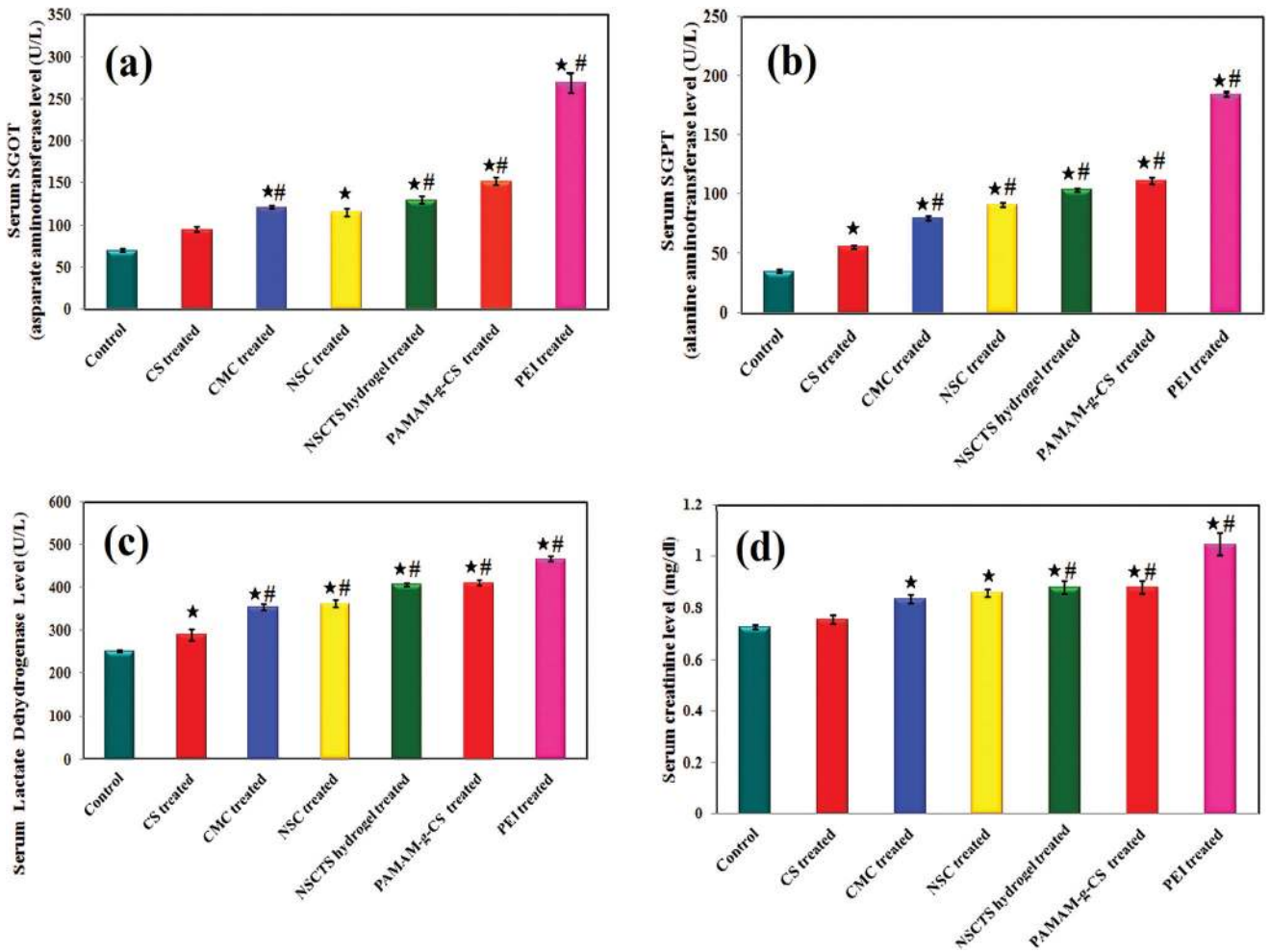

Fig. 3 Chronic toxicity study of modified chitosan treated animals, (a) serum SGOT, (b) serum SGPT, (c) serum LDH and (d) serum creatinine. Values are shown as mean ± SE (n = 6), star and # $p < 0.05$, significant.

waste products of the metabolism are removed by kidneys. Several essential homeostatic functions such as the regulation of electrolytes, regulation of blood pressure (*via* maintaining salt and water balance), maintenance of acid–base balance are performed by kidneys. They serve as a natural filter of the blood, removing water-soluble waste. In producing urine, the kidneys excrete waste such as urea and ammonium compounds, which are responsible for water, glucose, and amino acid re-absorption. Therefore, for the assessment of nephrotoxicity with chronic peroral treatment with modified chitosan, different parameters like the serum creatinine, urine creatinine and urine microprotein levels were measured. Measuring the serum creatinine level is the most commonly used indicator for renal function. A sharp rise in the blood creatinine level is noticed only with marked damage to the functioning nephrons. From Fig. 3d, it is noticed that the serum creatinine level of the control animal is 0.72 mg dl^{-1}, and for the CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS treated animals the value varies between 0.75–0.88 mg dl^{-1}. The serum creatinine reference range is 0.7–1.1 mg dl^{-1}. So, all the test values are within the reference range. Only in the case of the PEI treatment, the value of the serum creatinine level is observed to be a little higher (1.04 mg dl^{-1}). Again, the urine creatinine level was measured as an indicator for urinary tract obstruction, kidney failure, dehydration, severe kidney disease, shock, renal outflow obstruction and acute tubular necrosis. In comparison to the control animal, significant changes in the urine creatinine level (Fig. 4a) were also observed in the NSCTS hydrogel and PAMAM-*g*-CS treated groups. However, all values are within the reference range (8.4–24.6 mg kg^{-1} bw), indicating no nephrotoxicity. Proteins (albumin and globulin fractions) are generally involved in the maintenance of the normal distribution of water between blood and the tissues. Proteinuria may occur mainly in the case of increased glomerular permeability or in the case of defective tubular re-absorption. Therefore, the concentration of urine microprotein was assayed in the polymer treated animals in order to examine the renal disease or any glomerular damage. Fig. 4b shows that the concentration of urine microprotein in the control animal is 53 mg/24 h, whereas the CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS treated animals have urine microprotein levels ranging between 66–110 mg/24 h. A significant change is observed compared to the control group but all values are within the normal microprotein reference range of 28–140 mg/24 h, indicating no renal toxicity after peroral delivery of the chitosan derivates. The concentration of urea in urine is also

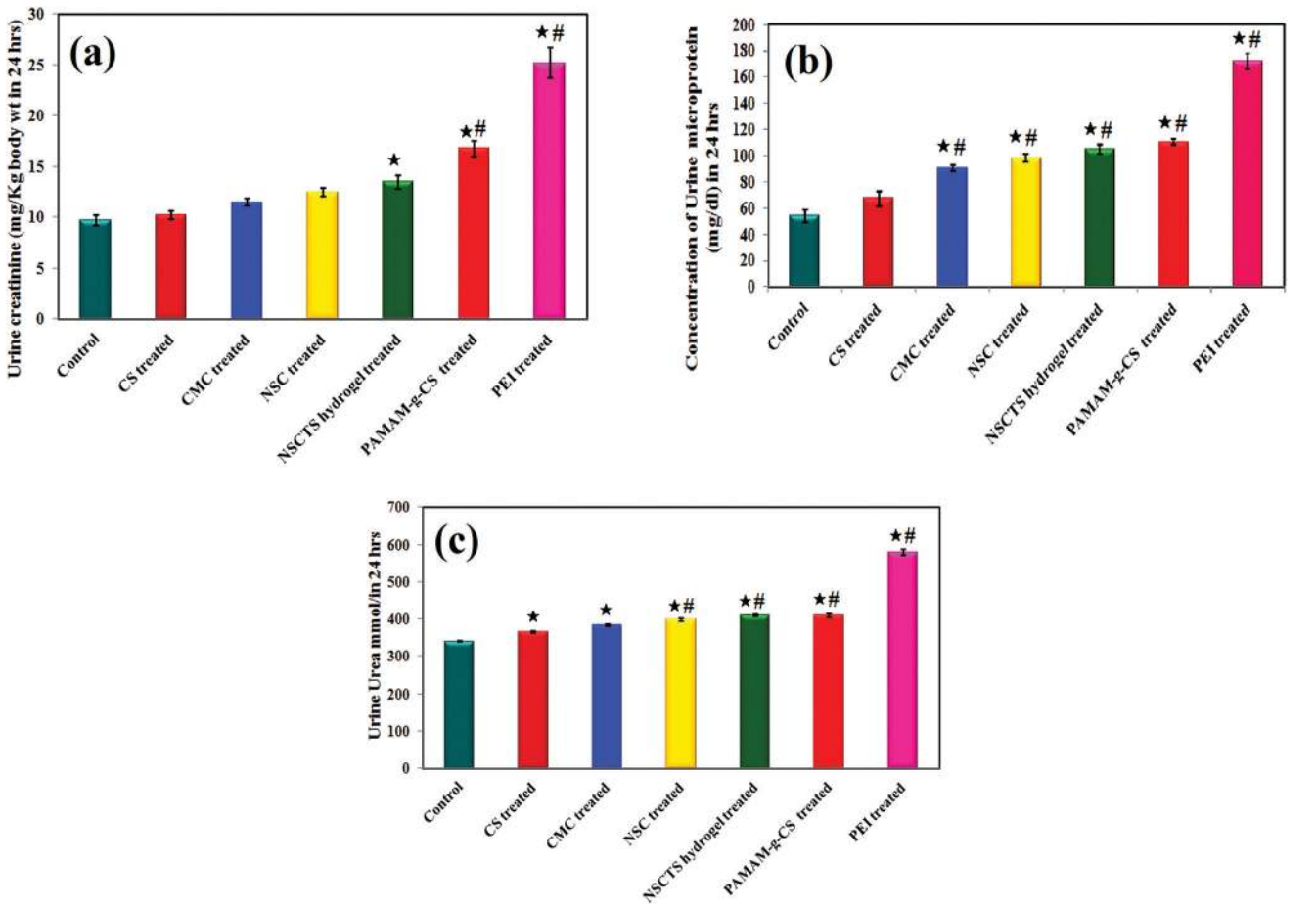

Fig. 4 Chronic toxicity study of modified chitosan treated animals, (a) urine creatinine, (b) urine microprotein and (c) urine urea. Values are shown as mean ± SE (n = 6), star and # $p < 0.05$, significant.

measured and presented in Fig. 4c. The urea concentration of the control group is 340.26 mmol in 24 h and for the polymer treated animals it ranges between 365–409 mmol in 24 h. A significant change is observed compared to the control animal group, although these values are within the reference range of 333–583 mmol/24 h. However, the PEI treated animals showed elevated creatinine, urea and microprotein levels (Fig. 4a–c). So, all these studies indicate that modified chitosan could be a safe alternative for oral insulin delivery, producing no renal dysfunction.

3.4. Pathohistological study

Pathohistological analysis was performed to observe the structural stability of the vital organs of the vertebrate body. The sections were grossly observed in H&E (hematoxylin and eosin) staining. The results of the microscopic observations of the liver and kidney sections (H&E staining) are shown in Fig. 5. The stomach and intestine were also examined. No ulcerative spot was noticed in the stomach after polymeric treatment as shown in Fig. 6. This suggested that the stomach is not affected by the polymers. In the pathohistological study (Fig. 5), the appearance of the liver sections upon treatment of the animals with CS, CMC, NSC, NSCTS hydrogel and PAMAM-*g*-CS was similar to the control tissue. The central vein with radiating hepatic cells was found in the control and treated sections, indicating no apparent hepatic toxicity of the polymers, whereas PEI caused a little damage to the liver found as nuclear debris and no clear central vein with radiating hepatic cells were observed. 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 the histological slides. The glomerulus, a tuft of capillaries, appears as a large cellular mass. These observations imply no renal toxicity after oral treatment with the polymers. But in the case of the PEI treatment, kidney damage was noticed (Fig. 5n). Again, the intestinal sections of the treated animals showed normal architecture, similar to the control tissue. However, a little damage to the intestinal epithelial cells and outer lining was observed (Fig. 6). The body weight and several behavioural parameters of the treated animals are grossly observed and presented in Table 1. Moreover, qualitative analysis of different biochemical parameters of urine (Table 2) showed no significant change after polymer treatment. Chitosan derivatives were prepared to improve the water solubility and pH sensitivity of native chitosan. Hence, several chemical modifications were implemented, which may have adverse effects within the animal system in long term diabetic treatment as these modified polymers would be used for formulating micro- or nanoparticles for efficient oral insulin delivery. It is suggested that no such adverse effects were found after administration of the polymer derivates to the animal body at a particular dose (30 mg kg^{-1}). So, the chronic toxicity study suggested that modified chitosan could serve as a promising and safe polymeric carrier for oral insulin therapy.

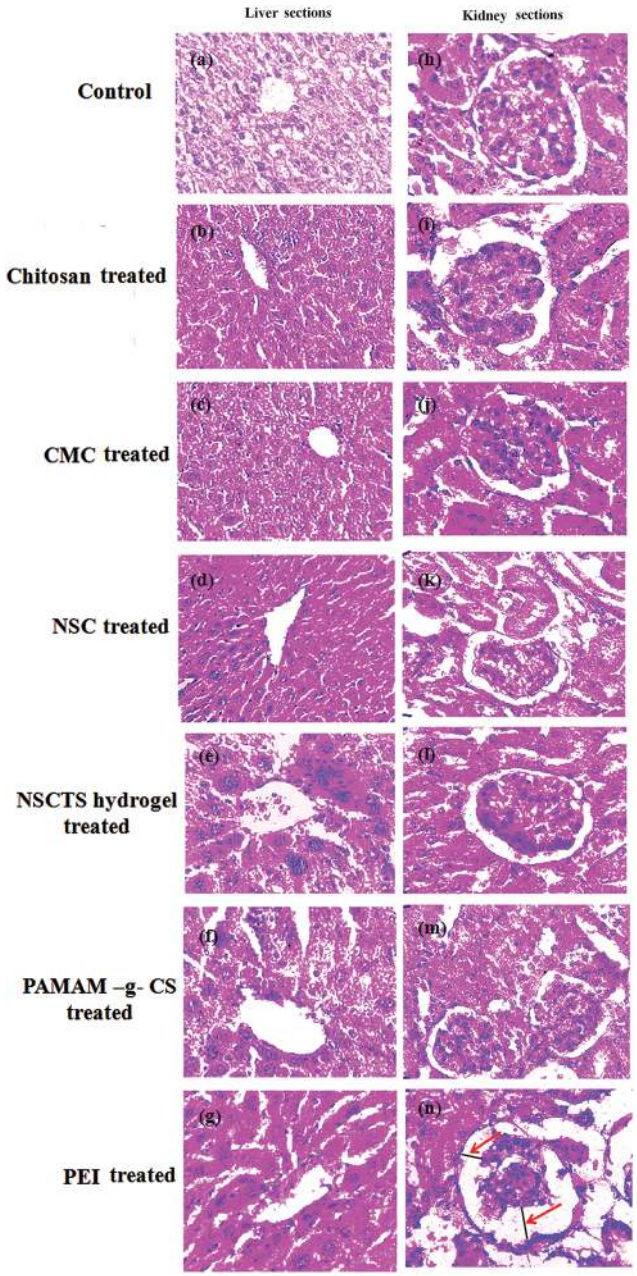

Fig. 5 Sections of liver and kidney (H&E staining, magnification 40×) of control and treated (peroral modified chitosan) mice.

3.5. Biodegradation of chitosan and its derivates

After examining the toxicity parameters in the chronic study, the polymers were subjected to a degradation study using microbes from the intestinal lumen, to ensure complete elimination as well as biodegradation of the carrier molecules after successful oral delivery of insulin.

3.5.1. Weight loss during biodegradation. Weight loss of the polymers through bacterial degradation signifies that the polymers, if used for oral insulin delivery, will be degraded by the natural microbial habitat of our body (Fig. 7a). In the study, we found that the susceptibility of the polymers to

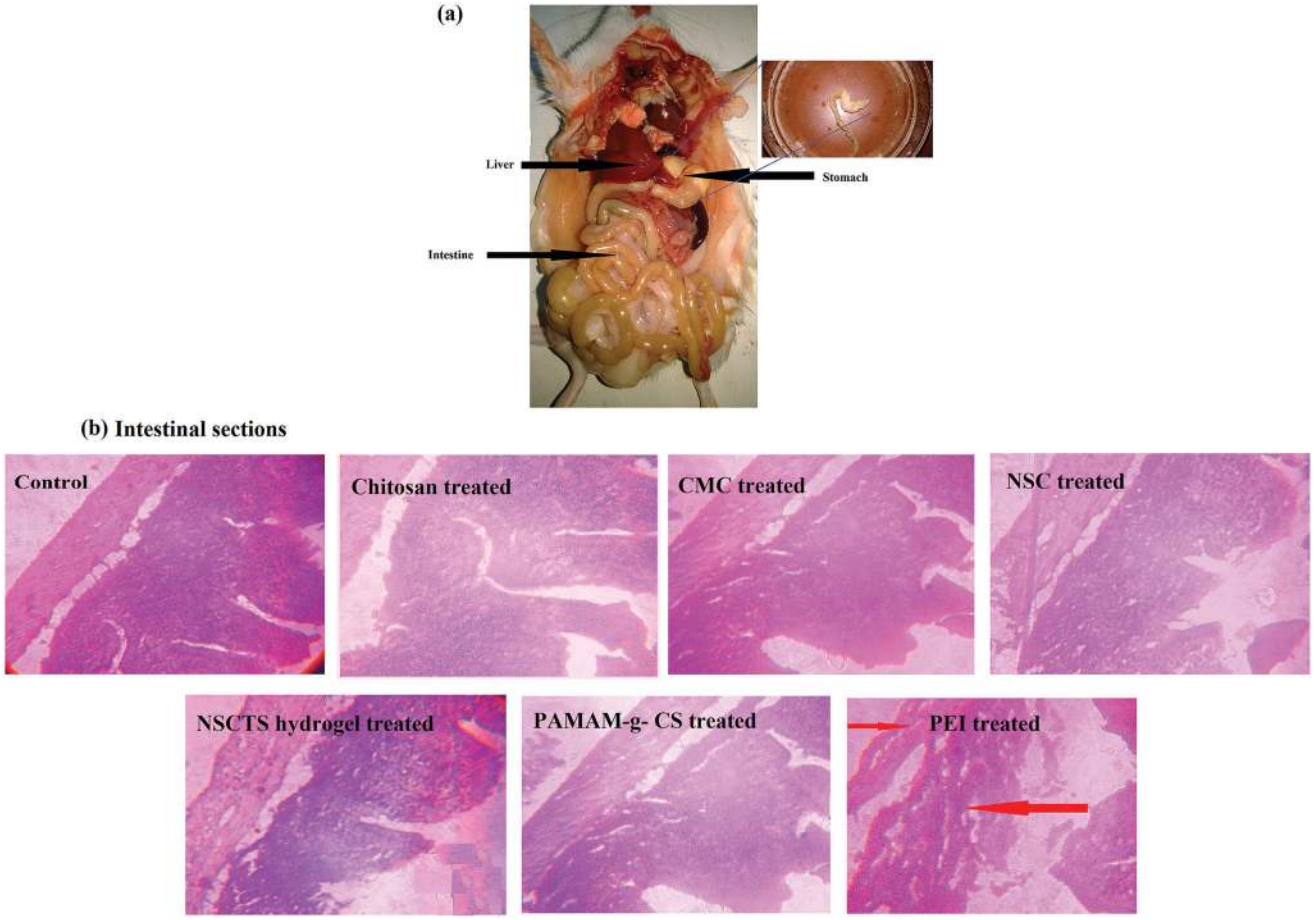

Fig. 6 (a) Internal organs of a treated animal, (b) sections of intestine (H&E staining, magnification 40×) of control and treated (peroral modified chitosan) mice.

Table 1 General appearance and behavioral observations of control and modified chitosan treated animals

	Control		CS treated		CMC treated		NSC treated		NSCTS hydrogel treated		PAMAM-*g*-CS treated		PEI treated	
Parameters	0 days	28 days	0 days	28 days	0 days	28 days	0 days	28 days	0 days	28 days	0 days	28 days	0 days	28 days
Average body weight (g)	28	29	26	28	26	25.5	28	28.3	26	28.4	27.2	29.3	28	24.5
Skin & fur	N[a]	N	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Behavioural pattern	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Food intake	N	N	N	N	N	N	N	N	N	N	N	N	N	Less
Salivation	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N	N	N	N	N	N	N	Lethr[b]
Sleep	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Coma	N.O[c]	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O
Tremors	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O	N.O

[a] N = Normal. [b] Lethr = Lethargic. [c] N.O = Not observed.

microbial degradation varied significantly. CMC was found to be highly biodegradable, showing 97.54% weight loss in 24 h. After that, it gradually started to dissolve, which made it impossible to measure its weight after 72 h. The two other rapidly weight losing polymers were PAMAM-*g*-chitosan (99.40% weight loss in 7 days) and NSC (98.38% weight loss in 7 days), whereas unmodified chitosan showed a comparatively lower degree of susceptibility to degradation (92.01% in 7

Table 2 Qualitative analysis of different biochemical parameters of urine in control and modified chitosan treated animals

Parameters	Control animal (0.9% NaCl treated)	Treatment with CS	Treatment with CMC	Treatment with NSC	Treatment with NSCTS hydrogel	Treatment with PAMAM-*g*-CS	Treatment with PEI
Urobilinogen	0.2	1	0.2	1	2	0.2	0.2
Protein	Negligible	Trace	Trace	Trace	Trace	Negligible	++
pH	7.5	7.5	8.0	7.5	7.5	8.0	8.0
Blood	Trace (non-haemolysed)	Trace (haemolysed)	Trace (haemolysed)	Trace (haemolysed)	Trace (haemolysed)	Trace (haemolysed)	Moderate (haemolysed)
Specific gravity	1.015	1.020	1.015	1.020	1.025	1.005	1.025
Ketone	15–30	Negligible	Negligible	Negligible	Negligible	Negligible	40–80
Bilirubin	Negligible	Negligible	Negligible	Negligible	+	Negligible	++
Glucose	Negligible	Negligible	Negligible	Negligible	Negligible	Negligible	Negligible

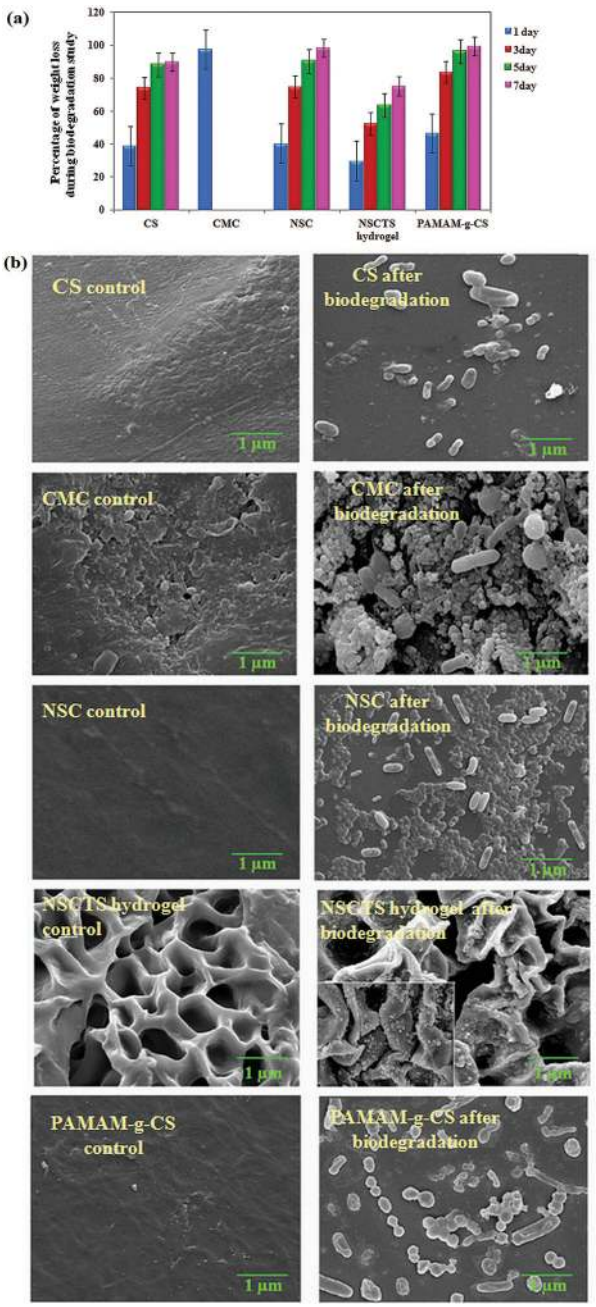

Fig. 7 Biodegradation of chitosan and its derivates: (a) weight loss during biodegradation, and (b) development of biofilm on the polymers.

days). The NSCTS hydrogel showed a relatively slow weight loss (75.21% in 7 days). As all polymers are biodegradable in nature, they are safe to be used for prolonged diabetes treatments by the oral delivery of insulin.

3.5.2. Biofilm development on the polymers. It is evident from the SEM images (Fig. 7b) that the polymers are favourable for microbial growth and development and, therefore, are susceptible to microbial degradation. It is observed that all the control polymers are devoid of any microbial growth, but the eventual development of biofilm on chitosan and the modified chitosan materials was observed. So, microorganisms play the key role in the biodegradation of polymers; this is also evident from the study of weight loss (Fig. 7a). It seems that the polymers are suitable substrates for the microbes as they break the –O–C–O– bonds present in the polymers.

4. Conclusions

The present investigation shows that chitosan derivates do not generate any chronic toxicity in *in vivo* animal models. No death or other signs of toxicity are observed in mice during the peroral treatment with different derivates of chitosan at a dose of 30 mg kg^{-1} per body weight for one month, thus establishing their safe application in oral insulin delivery. Again, in the pathohistological examination, no changes are observed in the architecture of the internal organs such as liver and kidney of the treated groups. Moreover, it is noticed that the intestinal luminal microbes cause successful biodegradation of these polymers (chitosan and their derivatives). Hence, it can be suggested that all the modified chitosan materials could serve as efficient and safe devices for the successful oral delivery of insulin and other therapeutic drugs in future.

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