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Evaluation of the biological properties of soluble chitosan and chitosan microspheres

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Abstract

Chitosan is the *N*-deacetylated product of chitin, a naturally occurring polymer which has been used extensively to prepare microspheres for oral and intra-nasal delivery. The chitosan polymer has also been proposed as a soluble carrier for parenteral drug delivery. Here the in vitro biocompatibility of seven soluble chitosan polymers (different salts and a chitosan derivative; glycol chitosan, different molecular weights and varying degrees of deacetylation) and chitosan microspheres loaded with horseradish peroxidase (HRP), was systematically studied by evaluating cytotoxicity towards B16F10 cells and ability to lyse rat erythrocytes. Cytotoxicity towards B16F10 was concentration-dependent and varied according to the salt used and polymer molecular weight. Chitosan hydrochloride was most toxic having an IC_{50} of 0.21 ± 0.04 mg/ml, only fourfold less toxic than the cationic reference polymer: poly-L-lysine (IC_{50} of 0.05 ± 0.01 mg/ml). The ranking of cytotoxicity was: chitosan hydrochloride > chitosan hydroglutamate > glycol chitosan > chitosan hydrolactate. Polymers of higher molecular weight of each type were most toxic. Glutaraldehyde-crosslinked chitosan microspheres were cytotoxic at all concentrations used. In the lysis assay, release of haemoglobin occurred on exposure to all soluble chitosans in a time-dependent manner. After 24 h, 100% lysis was conferred by most soluble chitosans (1 μg/ml–3 mg/ml), and even after 1 h, chitosan hydroglutamate caused 60–80% lysis. Microspheres were considerably less lytic than the soluble polymer. Scanning electron microscopy showed changes in B16F10 and red cell morphology consistent with the observed chitosan toxicity. Whilst HRP entrapped in glutaraldehyde crosslinked chitosan microspheres retained enzyme activity, this was a reduced 10-fold compared to an equivalent amount of native HRP. © 1997 Elsevier Science B.V.

Keywords: Chitosan; Biocompatibility; Microspheres; Drug targeting

1. Introduction

Chitin, or poly-β-(1-4)-*N*-acetyl-D-glucosamine

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(Fig. 1), is after cellulose the most abundant polymer found in nature, being a structural component of shellfish, insects and the cell walls of bacteria and mushrooms. Chitosan (Fig. 2) is the *N*-deacetylated product and has a molecular weight ranging from $1-3 \times 10^5$ Da. Due to its reported biocompatibility and biodegradability (Hirano et al., 1988), chitosan has been developed for a variety of biomedical applications including wound dressings and drug delivery systems. In the context of drug delivery, chitosan has been used as a stabilising constituent of liposomes (Henriksen et al., 1994); as an excipient controlling drug release in oral formulations (Imai et al., 1991; Kristl et al., 1993); as a nasal delivery system (Aspden et al., 1996); to prepare microspheres for encapsulation of enzymes, proteins and cells (Heller et al., 1996; Rha et al., 1984) and also to deliver DNA (Alexakis et al., 1995). In previous studies, (Carreño-Gómez et al., 1995; González-Siso et al., 1996) soluble chitosan hydrochloride (Seacure CL 210) was used to prepare, by spray-drying, glutaraldehyde-crosslinked microspheres of diameter 3–4 μm containing either horseradish peroxidase (HRP) or bovine serum albumin as model proteins. These microspheres are being developed as a system for oral vaccination.

Although it had been suggested that chitosan is a biocompatible polymer, preliminary studies led us to believe that soluble chitosan (like other cationic polymers) displayed concentration-dependent toxicity towards cells in vitro and also caused red blood cell lysis (Carreño-Gómez and Duncan, 1996). Thus, before pursuing further studies on chitosan as a component of drug or vaccine delivery systems, we decided it was important to study systematically the in vitro toxicity of soluble chitosans including polymers of different molecular weight and salt form, the soluble derivative, chitosan glycol, and in addition glutaraldehyde-crosslinked chitosan microspheres (Table 1). Biocompatibility of any biomaterial must be defined in the context of its intended route of administration, polymer form (soluble polymer, microparticle, fibre) and final use, including the likely frequency of dosing, therefore we considered it important to study both the soluble polymer as well as the microsphere.

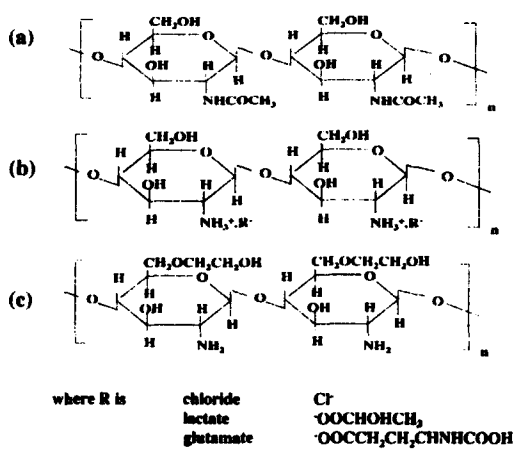

Fig. 1. Chemical structures of (a) chitin, (b) chitosan salts, and (c) glycol chitosan.

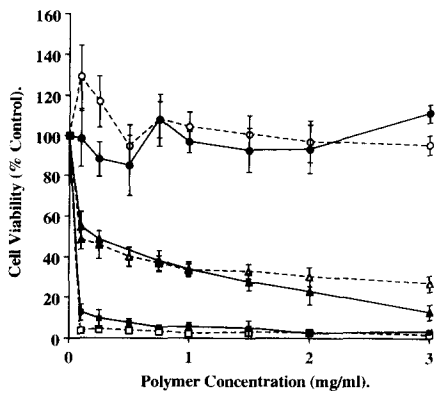

Fig. 2. Effect of soluble polymers on the viability of B16F10 cells in the presence and absence of serum. Results shown the effect of: dextran with serum (○---○); dextran without serum (●——●); CL210 with serum (△---△); Cl210 without serum (▲——▲); poly-L-lysine with serum (□---□) and poly-L-lysine without serum (■——■). Data represents the mean ± S.D. ($n = 24$).

Cytotoxicity of chitosans was assessed using a murine melanoma cell line, B16 F10, in combination with the MTT assay (Sgouras and Duncan, 1990) and their ability to cause lysis of rat erythrocytes in vitro was also studied. Throughout, scanning electron microscopy was used to monitor any changes in cell morphology. Furthermore, in context of the ultimate goal of the chitosan vaccine delivery system we monitored the retention of HRP activity once the protein was entrapped in microspheres using a standard spectophotometric assay (Schindler et al., 1976).

Table 1
Characteristics of the chitosans

Name	Code no.[a]	Viscosity (mPas)	Degree of deacetylation	pH (10 mg/ml in PBS)	Average molecular weight[b]
Hydrolactate	L 110	10	78	5.6	<50 K
	L 210	76	82	5.6	150–170 K
Hydroglutamate	G 110	13	>80	5.9	60–90 K
	G 210	76	77	5.8	180–230 K
Hydrochloride	CL 110	12	85	5.8	60–90 K
	CL 210	46	81	5.8	100–130 K
Glycol	none	(not supplied)	100	7.0	152 K

[a] Suppliers code.
[b] Number average molecular weight determined by viscosity measurements provided by supplier.

2. Materials and methods

2.1. Materials

Chitosan hydrochloride (CL110 and CL 210), chitosan hydroglutamate (G110, G210) and chitosan hydrolactate (L110 and L210) were supplied by Pronova (Norway). Glycol chitosan, dextran of M_w 74 000 (clinical grade) and poly-L-Lysine of M_w 56 000, horseradish peroxidase (HRP), bovine serum albumin (fraction V), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2.5% glutaraldehyde, 4% osmium tetroxide (VII) and hexamethyldisilazane (HMDS) (the latter four compounds were of electron microscopy grade) were all obtained from Sigma. See Table 1 for details of the chitosan products.

2.2. Evaluation of cytotoxicity

B16F10 cells (Prof. I. Hart, St. Thomas Hospital, London) were used to evaluate cytotoxicity. Cells in RPMI 1640 with or without 10% foetal calf serum were seeded into 96-well microtitre plates at a density of 1×10^4 cells/well. All chitosan polymers and chitosan microspheres were UV sterilised. Soluble chitosans, dextran or poly-L-lysine (the latter as reference controls) were added to the plates at various concentrations (0.1–3 mg/ml) in a final volume of 100 μl. After 72 h, 20 μl of MTT reagent was added and 5 h later the purple crystals produced were dissolved in 100 μl of optical grade dimethyl sulfoxide (DMSO). Absorbance was read at 550 nm using a microtitre plate reader.

To determine the cytotoxicity of chitosan microspheres (they interfered with the MTT assay) direct cell counting and trypan blue exclusion were used. Cells were seeded at 5×10^5 cells/well into 6-well cluster plates and microspheres added (0.1–3 mg/ml). After 72 h, 200 μl of trypan blue (0.2% w/v) were added and the viable cell number was determined using a haemocytometer slide.

2.3. Evaluation of red blood cell lysis

Blood was obtained from male Wistar rats after death by cardiac puncture. Erythrocytes were collected by centrifuging the blood three times in chilled phosphate buffered saline (PBS at 4°C) at $1000 \times g$ for 10 min. The final pellet was resuspended in PBS to give a 2% w/v solution. Using a microtitre plate assay, 100 μl of the erythrocyte solution was added to soluble chitosans (1 μg/ml–3 mg/ml) or chitosan microspheres (0.1–3 mg/ml) made up in a volume of 100 μl. Samples were then incubated for either 1 h or 24 h, the microtitre plate was centrifuged then at $1000 \times g$ for 10 min and the supernatants (100 μl) transferred into a new microtitre plate. Haemoglobin release was determined spectrophotometrically using a microtitre plate reader (absorbance at 550 nm). Dextran was used as the negative reference control and the detergent Triton X-100 (1% v/v)

used to produce 100% haemoglobin release. Results were expressed as the amount haemoglobin release induced by the polymers as a percentage of the total.

2.4. Scanning electron microscopy (SEM) studies

B16F10 cells were incubated with chitosan microspheres (not containing protein) or albumin-containing chitosan microspheres (both 1 mg/ml) for 24 h. The media was then removed and cells were fixed in 0.25% glutaraldehyde for 24 h, followed by 1% osmium tetroxide for 1 h. Samples were then dehydrated by incubation for 5 min in PBS solutions with increasing ethanol content. Finally they were dehydrated in HMDS and left to dry. Samples were gold-coated (Emtech, 20 μA) and visualised using a Phillips XL series scanning electron microscope (SEM).

Erythrocyte morphology was also visualised by using the same procedure after exposure to soluble chitosan CL 210 (1 mg/ml).

2.5. Determination of HRP activity

HRP activity was determined using a standard spectrophotometric assay (Schindler et al., 1976). Briefly, assays were conducted in phosphate-citrate buffer (0.05 M; pH 5.5) with 6 ng/unit of HRP (specific activity of HRP given as 1 mg of solid = 175–180 units) and varying concentrations ABTS. Final volume of phosphate-citrate buffer was 1 ml. Absorbance was read at 405 nm immediately after adding ABTS and samples were taken at different time points. In the case of HRP-containing microspheres the same procedure was used but 1 mg of chitosan microspheres was used, which at 10% HRP entrapment accounted for 18 units of the entrapped enzyme assayed.

3. Results

3.1. Effect of chitosan on B16F10 cells

A first cytotoxicity study was carried out using chitosan CL 210 in which the soluble polymer was added to cells in the presence or absence of serum (Fig. 2). It can be seen that addition of serum proteins made no significant difference (unpaired t-test) to the cytotoxicity observed; the concentration required to decrease cell viability by 50% (IC_{50}) was 0.2 ± 0.04 mg/ml in both cases. Therefore, all further the cytotoxicity experiments were carried out in serum-containing media.

All the chitosan salts tested (Fig. 3a–c) and chitosan glycol (Fig. 3d) displayed concentration-dependent cytotoxicity (Table 2). The polymers were less toxic than poly-L-lysine, but appreciably more toxic than the inert reference dextran. In contrast, the glutaraldehyde-crosslinked microspheres were extremely toxic (Fig. 4), even more so than poly-L-lysine in this assay.

SEM showed that B16F10 cells interact with chitosan microspheres (Fig. 5a), and particle internalisation was also observed, particularly for those containing albumin (Fig. 5b–d). After exposure to these microspheres morphology was very much altered, and plasma membrane rupture was typically seen as the cell became engorged with the protein laden particles (Fig. 5d).

3.2. Red blood cell lysis

After a 1-h incubation, only chitosan hydroglutamate caused extensive lysis (Fig. 6a), the extent of haemoglobin release being dependent on polymer concentration. When the incubation time was extended to 24 h, all the soluble chitosans (0–3 mg/ml) induced complete liberation of entrapped haemoglobin and polymer interaction with haemoglobin in solution actually caused it to precipitate making quantitation of lysis difficult. Over this time frame, all the soluble chitosans were highly membrane active. Even in the concentration range 1–100 μg/ml approximately 100% lysis was observed (results not shown). After exposure to soluble chitosan CL 210 (1 mg/ml) for 1 h, red cell aggregation and membrane rupture was visible by SEM (Fig. 7a–b), and at 24 h the red cell membrane was disrupted to the point of total lysis (Fig. 7c). These observations were consistent with the pattern of haemoglobin release observed.

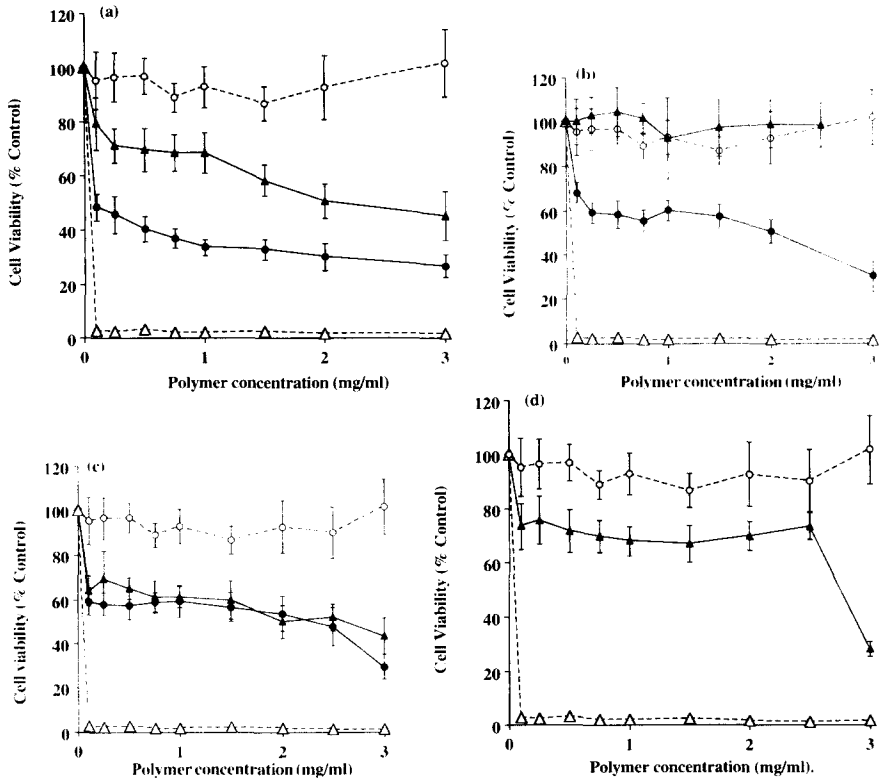

Fig. 3. Effect of different chitosan salts and chitosan glycol on the viability of B16F10 cells. All incubations carried out in the presence of serum. In each case dextran (○---○) and poly-L-lysine (△---△) were used as reference controls. Panel (a) shows chitosan chloride, CL110 (▲——▲) and CL210 (●——●); panel (b) shows chitosan lactate, L110 (▲——▲) and L210 (●——●); panel (c) chitosan glutamate, G110 (▲——▲), and G210 (●——●); and panel (d) glycol (▲——▲). Data represents the mean ± S.D. ($n = 24$).

3.3. HRP entrapment in chitosan microspheres

As protein entrapment in the glutaraldehyde-crosslinked microspheres caused a noticeable change in interaction with the B16F10 melanoma cells, it was considered important to quantitate the bioactivity of the enzyme. Table 3 shows the relative activity of free and entrapped HRP at different substrate concentrations. The calculated V_{max} and K_m for native HRP were 1.11 nM/s/unit and 0.015, respectively, whereas the values for entrapped HRP were 2.22×10^{-3} nM/s/unit and 0.144. Microsphere associated HRP was still showed some activity albeit highly reduced. This reduction is easily explained if one considers the harsh conditions of spray-drying where the temperature rises up to 110–120°C for a few seconds. In addition the glutaraldehdye will interact with the chitosan matrix and HRP, as it is well known to fix proteins.

Table 2
Cytotoxicity of soluble chitosan towards B16F10 cells

Chitosan	IC_{50}^{a} (mg/ml; mean ± S.E.)
Glycol chitosan	2.47 ± 0.15
L 110	2.50
L 210	2.00 ± 0.18
G 110	2.47 ± 0.14
G 210	1.73 ± 1.39
CL 110	2.24 ± 0.16
CL 210 (serum+)	0.21 ± 0.04
Cl 210 (serum−)	0.28 ± 0.04

[a] ($n = 24$).

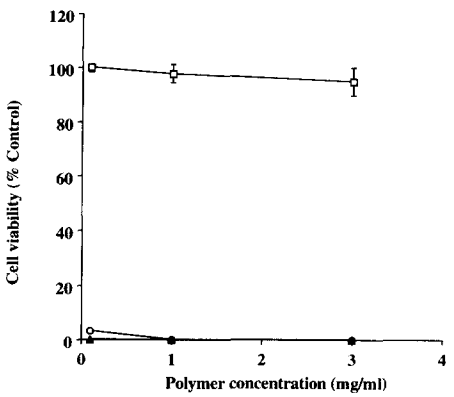

Fig. 4. The effect of chitosan microspheres on the viability of B16F10 cells. A comparison of dextran (□——□), poly-L-lysine (○——○) and chitosan microspheres is shown (▲——▲). Data represents the mean ± S.D. ($n = 6$).

4. Discussion

Although chitosan is widely described in the literature as 'biocompatible', non-toxic and biodegradable, few studies have, however, sought to document its basic biological properties for example define the in vitro IC_{50}. Studies rarely report the maximum tolerated dose when chitosan is administered by different routes in vivo. To date, most studies assume chitosan biocompatibility, and proceed to describe the performance of chitosan formulations designed for controlled release. From the data reported here it is clear that all the chitosan forms evaluated show some degree of toxicity (with the exception of L 110 in the B16F10 cytotoxicity experiments). Cytotoxicity was clearly related to the salt used, and this is not surprising as the nature of the counterion will govern interaction of the protonated amine function of the chitosan polymer with negatively charged cellular components. Chitosan hydrochloride (CL 210) was the most cytotoxic in the B16F10 assay, having an IC_{50} value of 0.21 ± 0.04 mg/ml and was only fourfold less toxic than poly-L-lysine (Sgouras and Duncan, 1990; Richardson et al., 1996). Chitosan cytotoxicity could be ranked in the order chitosan hydrochloride > hydroglutamate > glycol chitosan > hydrolactate. Polymer molecular weight also influenced toxicity, and all chitosans that had molecular weight of > 100 K (code 210, see Table 1 for details) were more toxic towards B16F10 cells.

Other studies have shown chitosan to be biologically active. Berscht et al. (1995) examined several different chitosans salts fabricated as fleeces destined for use as wound dressings. After a 72 h incubation with fibroblast cells, chitosan hydrolactate, hydrochloride and hydroglutamate inhibited cell growth by 70–80%. In contrast, a chitosan methylpyrrolidinone fleece caused only a 35% inhibition. Exposure of the same materials to fibroblasts during in their logarithmic growth phase resulted generally in less toxicity; chitosan methylpyrrolidinone fleeces reduced growth by 10%, hydrolactate by 21%, hydrochloride by 48%, but the chitosan hydroglutamate fleeces still caused a 76% inhibition. Although Kotzé et al. (1996) found that chitosan hydrochloride and hydroglutamate had no damaging effect on Caco-2 cells (MTT assay), the incubation duration was only 4 h.

We found that all the chitosans caused red blood cell lysis in a time- and molecular weight-dependent manner. At 1 h, relatively little lysis was seen except for the hydroglutamate chitosans, G 110 (75% lysis), G 210 (60% lysis) and hydrochloride Cl 210 (12%). However, over 24 h, lysis was so extensive that haemoglobin released into solution was actually precipitated, even at chitosan concentrations as low as 1 μg/ml. As the erythrocyte membrane contains anionic glycoproteins able to bind the protonated amino group of chitosan avidly; this induces membrane curvature, ultimately leading to rupture and haemoglobin release. The effect can be clearly visualised by SEM (Fig. 6a), after 1 h the erythrocyte membrane is already damaged, but at 24 h (Fig. 6b), cell aggregation and complete lysis is seen throughout. Chitosan interaction with blood components has been reported elsewhere (Lee et al., 1995). *N*-Acetyl chitosan (usually chitosan contains < 40% *N*-acetyl-D-glucosamine units) of molecular weight 1257 kDa and 674 kDa causes blood coagulation (times of 5.3 and 8.5 min, respectively, although derivatisation with *N*-hexanoyl groups reduces coagulation time to 17.2min. Hirano et al. (1988) showed that that intravenous injection of low molecular weight chi-

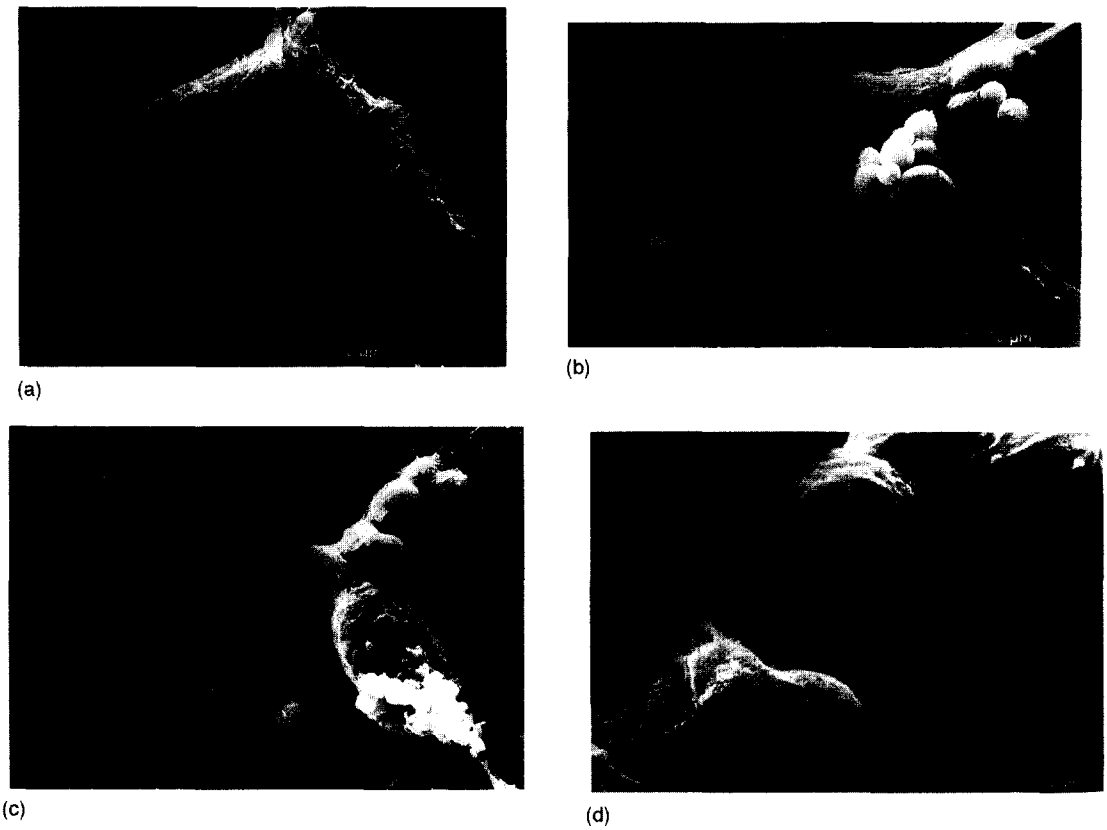

Fig. 5. Scanning electron microscopy of B16F10 cells after exposure to microspheres. Panel (a) control B16F10 cells with no microspheres; panel (b) B16F10 cells after incubation with microspheres not containing protein (1 mg/ml) for 24 h showing microspheres adherent to the cell surface; panel (c) B16F10 cells after incubation with microspheres containing albumin (1 mg/ml) for 24 h showing internalised microspheres; and panel (d) high power of panel (c) showing typical microsphere induced membrane damage.

tosan to rabbits at a dose of 4.5 mg/kg (approximately 10 mg/animal) daily for 11 days produced no abnormal changes, whereas a 50 mg/kg/day dose caused death after 3 days (one of two animals) and this was reported as probably due to blood cell aggregation. In this study it was also shown that oral dosing of chitosan for 239 days (0.7–0.8 g/kg) to rabbits or hens, produced little evidence of toxicity (loss of appetite occurred in the hens which was regained after chitosan feeding stopped).

Glutaraldehyde is the crosslinking agent most frequently used to prepare chitosan microspheres. It is obvious, however, that glutaraldehyde provides only a model system which will not be amenable to human use due to glutaradehyde-related toxicity. Our observations confirm extreme cytotoxicity of the glutaraldehyde-crosslinked chitosan microspheres in the B16F10 model. This effect could be due to residual bioreactive aldehyde groups combined with damage induced by chitosan itself. Jamaela et al. (1994) used glutaraldehyde crosslinked chitosan microspheres as carriers for diphtheria toxoid and albumin and tested their behaviour in vivo. Six months after intramuscular injection, the microspheres had not degraded and histological studies revealed a moderate cellular immune response with presence of macrophages and giant cells. Improved microsphere biocompatibility can be achieved with

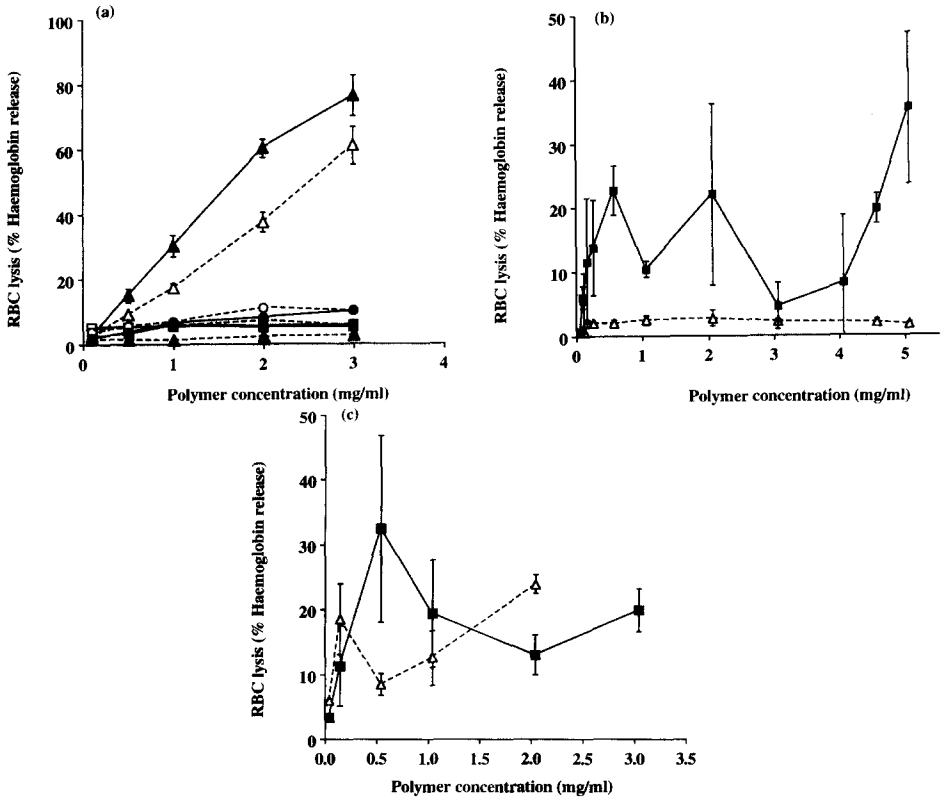

Fig. 6. Haemoglobin release after incubation of rat RBCs with soluble chitosan or chitosan microspheres. Panel (a) lysis after incubation with soluble chitosans for 1 h; chitosan G110 (▲——▲) and G210 (△- - -△); L110 (■——■) and 210 (□- - -□); CL 110 (○- - -○) and 210 (●——●); glycol chitosan (▲- - -▲) and dextran (□——□); panel (b) lysis after incubation with chitosan microspheres for 1 h, microspheres (■——■), dextran (△- - -△); and panel (c) lysis after incubation with chitosan microspheres for 24 h, microspheres (■——■), dextran (△- - -△). In all cases data represents the mean ± S.D. ($n = 12$).

other crosslinking such as citric acid (Orienti et al., 1996), and alginates (Heller et al., 1996). Cytotoxicity tests performed in our laboratory (using the same tests as described here) showed excellent biocompatibility of the alginate-microspheres of Heller et al. (1996).

B16F10 cells readily phagocytose chitosan microspheres and this is clearly seen in the SEM pictures. There was increased internalisation of the albumin-containing particles suggesting a role of protein in triggering uptake. After microsphere internalisation the cell morphology was drastically altered and in some cases membrane rupture was observed suggesting that cells were not able to quickly degrade the particle and consequently membrane lysis was induced. In the red blood cell assay, the chitosan microspheres were considerably more lytic than dextran, they were considerably less membrane active than the soluble chitosans.

The fact that HRP maintained some activity, albeit highly reduced, suggests that biologically active protein, or antigen, could be entrapped in such structures. Whereas the control chitosan microspheres have in cross-section, a thin, smooth surface, incorporation of protein alters the morphology in that small protein particles are visible on the surface. It is possible that this is responsible for the preserved enzyme activity, but, Alexakis et al. (1995) suggested that chitosan microspheres are glutaraldehyde crosslinked only in the outer shell, and not deep within the core of the microsphere. If the enzyme substrate penetrates this could also explain the residual HRP

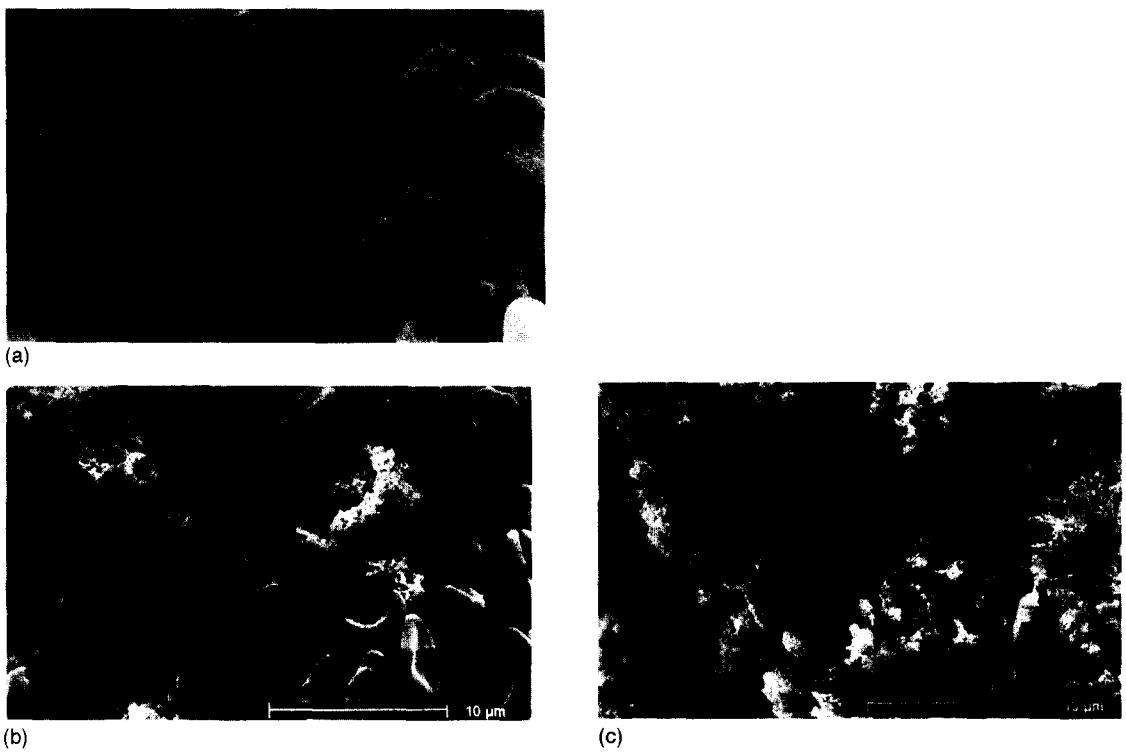

Fig. 7. Scanning electron micrographs of rat RBCs after exposure to soluble chitosan. Panel (a) control RBCs; panel (b) after exposure to CL 210 for 1 h; and panel (c) after exposure to CL 210 for 24 h.

activity. González-Siso et al. (1996) showed activity of invertase and α-amylase in the same chitosan-glutaraldehdye microspheres and concluded that amount of enzyme activity preserved was dependent on degree of crosslinking degree.

Table 3
HRP activity in native form and entrapped in chitosan microspheres

ABTS (μg/ml)	Initial rate[a]	
	Free HRP	Microspheres HRP
10	0.138	1.645×10^{-4}
20	0.275	2.791×10^{-4}
40	0.564	4.967×10^{-4}

[a] Initial rates (nmol/unit/s).

In conclusion, chitosan polymers when used as soluble polymeric carriers for intravenous administration, or when liberated following microparticle catabolism have the potential to induce cellular toxicity. It is essential that more studies are carried out to document chitosan formulations in models relevant to the clincial setting in which they will ultimately be used. Careful selection of the chitosan salt used (we found the hydrolactate the least toxic) and polymer molecular weight will improve chances of good biocompatibility. Already we have found in a related study, that the soluble chitosan used to fabricate alginate crosslinked particles was non-cytotoxic and non-lytic (Heller et al., 1996). The data reported here suggest that soluble chitosans of high molecular weight would not make ideal candidates for intravenous administration.

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