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Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA

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Abstract

Cationic polymers have the potential for DNA complexation and it is recognised that they may be useful as non-viral vectors for gene delivery. Highly purified chitosan fractions of < 5000 Da (N1), 5000–10 000 Da (N2) and $> 10\,000$ Daltons (N3) were prepared and characterised in respect of their cytotoxicity, ability to cause haemolysis, ability to complex DNA as well as to protect DNA from nuclease degradation. Also the biodistribution of ^{125}I-labelled chitosans was followed at 5 and 60 min after intravenous injection into male Wistar rats. All chitosan fractions displayed little cytotoxicity against CCRF-CEM and L132 cells ($IC_{50} > 1$ mg/ml), and they were not haemolytic ($< 15\%$ lysis after 1 and 5 h). Chitosan–DNA interaction at a charge ration of 1:1 was much greater than seen for poly(L-lysine) and complexation resulted in inhibition of DNA degradation by DNase II: 99.9 ± 0.1, 99.1 ± 1.5 and $98.5 \pm 2.0\%$ for N1, N2 and N3, respectively. After intravenous injection, all the chitosans showed rapid blood clearance, the plasma levels at 1 h being $32.2 \pm 10.5\%$ of recovered dose for N1 and $2.6 \pm 0.5\%$ of recovered dose for N3. Liver accumulation was molecular mass dependent, being $26.5 \pm 4.9\%$ of the recovered dose for N1 and $82.7 \pm 1.9\%$ of the recovered dose for N3. The observations that the highly purified chitosan fractions used were neither toxic nor haemolytic, that they have the ability to complex DNA and protect against nuclease degradation and that low molecular weight chitosan can be administered intravenously without liver accumulation suggest there is potential to investigate further low molecular weight chitosans as components of a synthetic gene delivery system.

Keywords: Biocompatibility; Body distribution; Chitosan; Gene delivery

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1. Introduction

Increasing understanding of the molecular basis of disease provides the opportunity to modulate, or replace, a dysfunctional gene. Development of gene therapy into routine clinical practice will depend upon our ability to design vectors that are suitable for scale-up manufacture, amenable to repeated systemic administration and that also have the ability to target, and efficiently transfect, cells in vivo (Felgner, 1990; Kabanov and Alakhov, 1993; Crook, 1995; Douglas and Curiel, 1995; Lasic and Templeton, 1996). Transfection techniques used in vitro include electroporation, calcium phosphate co-precipitation of DNA and the 'gene gun', which can shoot particles into cells at high velocity (Fynan et al., 1993). All of these approaches are, however, unlikely to have wide application in vivo.

Systems currently under study for in vivo use include both viral (Morgan and Anderson, 1993) and non-viral constructs (Ledley, 1994; Zelphati and Szoka, 1996). Viral vectors are very effective in terms of transfection efficiency, but they have limitations in vivo including wild-type reversion and immunogenicity (Wilson et al., 1990; Douglas and Curiel, 1995; Verma and Somia, 1997). Non-viral delivery systems include cationic lipids (Kabanov and Alakhov, 1993; Zelphati and Szoka, 1996) and cationic polymers. Cationic lipids, such as Transfectam (Behr et al., 1989), provide effective synthetic transfection systems, but their use in vivo is limited by general toxicity, complement activation and liver and lung tropism.

Since the studies with poly(L-lysine) in the late 1980s (Wu and Wu, 1987) many cationic polymers have been explored as non-viral vectors. These include polyethyleneimine (Boussif et al., 1995; Remy et al., 1998), polybrene (Mumper et al., 1996) and poly(amidoamine) (PAMAM) dendrimers (Tang et al., 1996). Neutral polymers interact with DNA via hydrogen bonding, and polyvinylpyrrolidone and poly(vinyl alcohol) (Mumper et al., 1998) have been explored as gene delivery vectors. To date the transfection efficiency of such polymers has been relatively poor and many of the cationic polymers that have been investigated are relatively toxic, with little potential for repeated intravenous administration. Several empirical factors must be considered when designing polymeric gene delivery constructs for in vivo use. The polymeric vector must be: biocompatible (non-toxic and non-immunogenic, and preferably biodegradable); able to complex DNA to form small particles which can be formulated reproducibly; able to protect the complexed DNA from degradation during transport in the bloodstream; able to deliver DNA to the target tissue in sufficient quantity; and, ideally, capable of targeting particular cell types. At the cellular level, the polymeric vector must be able to deliver the DNA payload to the cytoplasmic compartment of the cell. This usually involves pinocytic capture of the polymer–DNA complex with subsequent transfer across the endosomal or lysosomal membrane into the cytosol. Once inside the cell, the construct must allow liberation of DNA to ensure nuclear translocation and regulated gene expression.

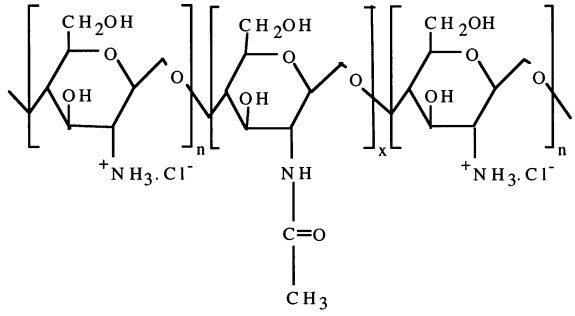

Fig. 1. Chemical structure of chitosan.

Here we have studied systematically several of the properties of low molecular weight chitosan fractions (Fig. 1 and Table 1) that are considered important in respect of their potential use in a

Table 1
Physical properties of the chitosan molecular mass fractions

Chitosan	Degree of deacetylation (%)	Molecular mass (Da)
N1	65.4	<5000
N2	55.3	5000-10000
N3	55.3	>10 000

DNA delivery system. Chitosan is a naturally occurring polysaccharide produced by deacetylation of chitin (Skaugrud, 1989; Aspden et al., 1996); it is composed of monomer units of *N*-acetylglucosamine and glucosamine linked by β1,4-glycosidic bonds. Initially the cytotoxicity and haemolytic properties of the chitosan fractions were examined using assays that had previously shown chitosans of high molecular weight and specific salt form to be toxic (Carreno-Gomez and Duncan, 1997). The ability of the chitosans to form complexes with DNA was studied using agarose gel electrophoresis and the ability of complexed polymers to stabilise DNA was examined by assessing degradation in the presence of DNase II. To follow biodistribution, chitosan fractions of different molecular mass were radioiodinated using the Bolton and Hunter reagent and the tissue distribution measured at 5 min and 1 h after intravenous injection to rats.

2. Materials and methods

2.1. Materials

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer-tyrosinamide ($TyrNH_2$) of Mw ≈ 20 000 Da was used as a reference polymer for the biodistribution studies and was prepared and characterised as described previously (Duncan et al., 1984). Fetal calf serum (FCS), E199 and RPMI 1640 medium were supplied by Gibco (UK). 3-[4,5-Methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), optical grade dimethylsulphoxide (DMSO), Triton X-100, dextran (72 000 Da), poly(L-lysine) (56 000 Da), λ *Hin*dIII DNA digest, chloramine T, calf thymus DNA and DNase II were all supplied by Sigma (UK). Bolton and Hunter reagent and sodium [^{125}I]iodide were supplied by Amersham International (UK). Human embryonic lung (L132) and human lymphoblastic leukaemia (CCRF-CEM) cell lines were from ECCAC (UK). Wistar rats were supplied by Banton and Kingman (UK). Oxygen (medical grade) and isofluorothane were supplied by Abbott Laboratories (UK).

2.2. Preparation of chitosan molecular mass fractions

Chitosan (25 g; Fluka, ref. 22741) was placed in a 1-l glass bottle equipped with a magnetic stirring bar and 4 M HCl (625 ml) was added. The gas phase was thoroughly purged with nitrogen and the reaction bottle closed tightly. The bottle was placed in an oil bath, set at 100°C, and the chitosan solution was vigorously stirred and kept at 100°C for 4 h. The reaction mix was poured through a plastic sieve (to remove residual chitosan particles) into a 2-l glass bottle. The bottle was closed and placed in ice water. After cooling, 625 ml of ethanol were added and the mixture left at 2–4°C overnight. Precipitated material was centrifuged in the cold (10 000 × *g*, 10 min) and the supernatant discarded. The pellets were washed with 400 ml of cold 50% ethanol in Milli Q water (v/v) and the supernatant was discarded. The washing step was repeated. The pellet was resuspended in 20–60 ml of Milli Q water, frozen and lyophilized.

Usually chitosan fractions of 12–16 g were obtained and these can be stored in dried form. To remove pigments, 14.9 g of the chitosan fraction was dissolved in 300 ml of Milli Q water and the aqueous phase first extracted with 100 ml of 2-butanol and then with 100 ml of 50% 2-butanol in hexane (v/v); both organic phases were discarded. The aqueous phase was concentrated by rotary evaporation in vacuum. The remaining pellet was dissolved in 50–100 ml of Milli Q water and ultrafiltered using a YM-5 membrane (Amicon). The concentrate (10–20 ml) was washed three times (reconcentrated) with 50–100 ml of Milli Q water. The pooled filtrates were frozen and lyophilized: a white powder, chitosan fraction N1 (< 5 Da) was obtained. The same procedure was carried out for the remaining concentrate using a YM-10 membrane (Amicon). The pooled filtrates were frozen and lyophilized; a white powder, chitosan fraction N2 (5–10 Da), was obtained. The remaining concentrate was frozen and lyophilized; a slightly yellowish powder, chitosan fraction N3 (> 10 Da), was obtained. The material was stored in brown bottles in the dark.

2.3. Evaluation of cytotoxicity

Before all in vitro experiments were carried out, the effect of the polymer on the pH of the respective media was investigated. Chitosan concentrations below 100 μg/ml did not alter the pH. L132 cells in E199 media containing 5% FCS were seeded into 96-well microtitre plates at a density of 5×10^4 cells/well. CCRF-CEM cells in RPMI 1640 media containing 10% FCS were seeded at a density of 5×10^4 cells/well into 96-well V-bottomed microtitre plates. In both cases, cells were incubated for 24 h prior to the addition of 0.2-μm-filter-sterilised polymer. Dextran and poly(L-lysine) were used as negative and positive reference, respectively. The MTT assay was used to determine cell viability (Twentyman and Luscombe, 1987). Cells were incubated with polymer for 67 h before the addition of the MTT (20 μl/well at a concentration of 5 mg/ml in phosphate-buffered saline (PBS). After a further incubation period of 5 h, the cells in suspension were centrifuged, the media removed and DMSO (100 μl) was added and left for 1 h. The absorbance was then read at 550 nm using a Titertek plate-reader. Results are expressed as the percentage viability with respect to control cell cultures with no polymer added. For cells grown as a monolayer, the same protocol was followed with the omission of the centrifugation step (Sgouras and Duncan, 1990).

2.4. Evaluation of haemolysis

Chitosan fractions, and dextran, poly(L-lysine) or Triton X-100 (1%, v/v) as controls, were diluted in PBS and mixed with an equal volume of a suspension (2%, w/v) of fresh, rat red blood cells (RBCs). After incubation at 37°C for 1 h or 5 h, the preparation was centrifuged at $1500 \times g$ for 10 min at room temperature. Then 100 μl of the supernatant were removed and analysed spectrophotometrically at 550 nm for haemoglobin release. The results were expressed as the percentage haemoglobin released relative to the release caused by Triton X-100 (Duncan et al., 1991).

2.5. DNA–polymer interactions: gel retardation assay and DNase degradation assay

Chitosan fractions, dextran or poly(L-lysine) were mixed with λ *Hin*dIII DNA on a charge ratio basis, except in the case of dextran where a weight ratio was used. Complexation was performed in 0.9% sterile saline at room temperature, the preparation being left to stand for 30 min prior to analysis. The assay used an agarose gel (0.5%, w/v) containing ethidium bromide (0.25 μg/ml) in tris-acetate/EDTA (TAE) buffer run at 100 V for 1 h. A λ *Hin*dIII digest was used as a molecular mass marker and the pattern of banding was visualised by UV transillumination and photographed using a Polaroid land camera (667 film).

DNase II degradation of calf thymus DNA was assayed as previously described (Barret and Heath, 1977). Calf thymus DNA (100 μg/ml) and the DNA–polymer complexes were incubated with DNase II (300 units/ml) in sodium acetate–acetic acid buffer (0.2 M, pH 5) containing potassium chloride (0.2 M) at 37°C. At various times up to 1 h, samples (0.5 ml) were taken and precipitated with 10% (w/v) perchloric acid (0.5 ml). After 20 min at 4°C, the samples were centrifuged at $12\,000 \times g$ for 20 min and the absorbance of the supernatant was measured at 260 nm. Results were expressed as percentage control degradation at 60 min.

2.6. Preparation of ^{125}I-labelled polymers

The chitosan fractions were radiolabelled using the Bolton–Hunter reagent (Bolton and Hunter, 1973). Polymers were first dissolved at 10 mg/ml in borate buffer (0.1 M, pH 6.5) and converted to the free base by addition of trimethylamine (97.5 μl of triethylamine per 5 mg of polymer in 500 μl of borate buffer). The solvent (dry benzene–2% dimethylformamide) was then removed from Bolton–Hunter reagent (100 μl, 500 μCi) under a stream of nitrogen in a fumehood. The chitosan solution (500 μl) was then added to the dry Bolton–Hunter reagent and stirred on ice for 15 min. Then 5 μl of the reaction mixture were removed for determination of labelling efficiency

by paper electrophoresis. The remaining reaction mixture was placed in dialysis tubing (molecular mass cut-off 1200 Da) and dialysed against 1% (v/v) HCl until no radioactivity was found in the dialysate. The quantity of free [^{125}I]iodide remaining in the preparation was then assessed by paper electrophoresis.

HPMA copolymer-TyrNH_2 (Duncan et al., 1984) was radiolabelled by the Chloramine T method. HPMA copolymer-TyrNH_2 (5 mg) was dissolved in 0.5 ml of 0.01 M phosphate buffer (pH 7.0) and placed on ice. Then sodium [^{125}I]iodide (5 μl, 500 μCi) was added under stirring and allowed to stand for 2 min. Chloramine T solution (2 mg/ml, 75 μl) was added and left to stir for 15 min. Then sodium metabisulphate (2 mg/ml, 500 μl) and a crystal of KI were added and the mixture was stirred for 2 min. A sample of the reaction mixture was taken to determine the labelling efficiency. The reaction mixture was placed in dialysis tubing (1200-Da cut-off) and the product dialysed against 1% NaCl. The specific activity and purity of the product were then determined by paper electrophoresis as before. All samples were stored at 4°C.

2.7. Body distribution of polymers after intravenous injection

For each polymer, 1×10^6 counts/min (cpm) were diluted into 1.5 ml of 0.9% NaCl. If necessary, the solution was neutralised with saturated NaOH and the final volume was then made up to 2 ml. From this stock, 200 μl ($\approx 5 \times 10^5$ cpm) of polymer were injected into the tail vein of adult male Wistar rats ($\approx$ 250 g) anaesthetised with a mixture of 4% (v/v) oxygen and 2% (v/v) isofluorothane in air. Animals were placed in metabolic cages for 1 h and then killed using CO_2 asphyxiation. Each animal was weighed and the major organs were removed, weighed and dissolved in 5 or 10 ml of 10 N NaOH containing 0.3% (v/v) Triton X-100. The following tissue samples were taken: liver, lungs, heart, kidneys, spleen, thyroid and urine. Blood samples (1 ml) were also taken. With the exception of blood and urine, the samples were then left to digest for 5 days or until the organs had solubilised. Samples (1 ml) were then assayed for radioactivity using a gamma counter (LKB Wallac) in replicates of three (three samples per organ per rat.). The results were expressed as percentage of dose for each organ and as a percentage of the recovered dose. The blood volume of the rat was calculated assuming 7.2 ml blood per 100 g of rat (Duncan et al., 1982).

3. Results and discussion

Cationic polymers have frequently been selected for use as non-viral vectors. They are an attractive choice as simple mixing with DNA in vitro leads to electrostatically driven self-assembly of polymer–DNA complexes into nanoscale polyplexes (for terminology, see Felgner et al., 1997). Frequently these particles are very heterogeneous in nature. The cationic polymers most often studied, including poly(L-lysine) (Wu and Wu, 1987; Zauner et al., 1998), polyethyleneimine (Remy et al., 1998) and PAMAM dendrimers (Tang et al., 1996; Malik et al., 1997), are very toxic to cells. Although DNA complexation, with the consequent charge neutralisation, abrogates this toxicity, it is nonetheless a concern when one considers the ultimate fate of the construct and possibility for localised liberation of the toxic polycation, hence the need to identify non-toxic polycations for use as synthetic DNA vectors.

Due to its use in oral and topical preparations, chitosan has been widely reported as a 'biocompatible polymer' (e.g. Hirano et al., 1988; Bersch et al., 1995), but there is frequently neither experimentation to support the claim nor a clear specification describing the physicochemical characteristics of chitosan used. It should be stressed that the accepted definition of material biocompatibility demands that the term 'biocompatible' must be qualified in relation to the intended use, route of administration and frequency of administration (Williams, 1987). 'Chitosans' can be prepared in a variety of physical forms: soluble polymers, hydrogels, microparticles, fibres and tablets. The parent polymer can also have a wide range of molecular weight and degree of deacetylation, and may be used in a variety of

different salt forms. In relation to their potential use for systemic administration, we have previously studied haemolysis and cytotoxicity of commercially available chitosans (Carreno-Gomez and Duncan, 1997). Using standardised techniques, extensively calibrated using a variety of polymers as standards (Sgouras and Duncan, 1990), it was found that a variety of chitosans were cytotoxic and haemolytic, the extent being dependent upon their molecular weight, degree of deacetylation and salt form. Toxicity increased with increasing molecular weight and chitosan hydrochloride was the most toxic form (IC_{50} of 0.21 ± 0.04 mg/ml), being only fourfold less toxic than the cationic reference polymer poly(L-lysine) (IC_{50} 0.05 ± 0.01 mg/ml). The ranking of cytotoxicity was: chitosan hydrochloride > chitosan hydroglutamate > glycol chitosan > chitosan hydrolactate (Carreno-Gomez and Duncan, 1997). Subsequent studies using chitosan of lower molecular weight showed that toxicity could be substantially decreased (Heller et al., 1996) and the purified, low molecular mass fractions of chitosan studied here were found not to be toxic against L132 human embryonic lung cells (Fig. 2a) or CCRF-CEM human lymphoblastic leukaemia cells (Fig. 2b) up to concentrations of 500 μg/ml. In contrast, poly(L-lysine) was toxic at all concentrations above 50 μg/ml. Little haemolysis (< 15%) was observed at 1 h (Fig. 3a), and increasing the incubation time to 5 h only increased the extent of lysis observed for poly(L-lysine) (Fig. 3b). These observations suggest that these chitosan fractions are suitable for intravenous administration.

All the chitosan molecular mass fractions readily complexed DNA (Fig. 4a), even down to a DNA:chitosan charge ratio of 1:0.01. This complexation was considerably more efficient than that seen for poly(L-lysine) of different molecular mass and also dextran (Fig. 4b). Polymer–DNA complexation is known to be influenced by many factors such as salt concentration, pH, polymer charge density, polymer molecular weight, polymer tertiary structure and the polymer to DNA charge ratio (Boussif et al., 1995; Kabanov and Kabanov, 1995). Both electrostatic interactions and hydrogen bonding play a role in complexation.

For effective in vivo delivery, it is essential that a synthetic vector can stabilise DNA during transport to the target cells. Preparation of chitosan–DNA complexes (at all charge ratios) resulted in a significant decrease in degradation by DNase II (Fig. 5a–c). At a charge ratio of 1:1 no degradation was seen, and even at a charge ratio of 1:0.05 there was approximately 80% less degradation at 60 min (Fig. 5c) The DNase II enzyme was selected as a model system; it is known to act by cleavage of both DNA strands leaving the 3′-phosphate end-group associated with the nucleotide (Barret and Heath, 1977). It is probable that protection arises as complexation-induced changes in DNA tertiary structure causing steric hindrance. It has been shown that addition of poly(L-lysine) to plasmid DNA causes a large change in tertiary structure visible using scanning electron microscopy (Wagner et al., 1991), and also toroid formation (Hud et al., 1995).

To determine the inherent biodistribution of chitosans of different molecular mass, ^{125}I-labelled samples were prepared using the Bolton–Hunter reagent. The labelling efficiency of these samples was adequate, giving preparations of specific activity > 0.3 μCi/mg and of high purity (< 1.8% free [^{125}I]iodide). After intravenous injection, ^{125}I-labelled fractions N2 and N3 were subject to particularly rapid plasma clearance (Fig. 6c,d); less than 15% of the dose recovered at 5 min was present in the blood and more than 50% of the recovered dose was already in the liver. The lowest molecular mass fraction N1 was cleared from the circulation more slowly (Fig. 6b), with 30% of the recovered dose present in the blood, and less than 30% in the liver, at 60 min. ^{125}I-labelled HPMA copolymer (Fig. 6a) had the expected body distribution at both time points (Duncan et al., 1982), showing a considerably longer plasma residence time than the chitosans and low levels of liver accumulation. It is important to note that the recovery of ^{125}I-labelled polymer recovered from the organs sampled varied significantly (Table 2). The relatively low recovery of ^{125}I-labelled chitosans is consistent with that seen for other carbohydrate polymers and may be indicative of rapid extravasation or interaction with endothelial cells. Further experiments including

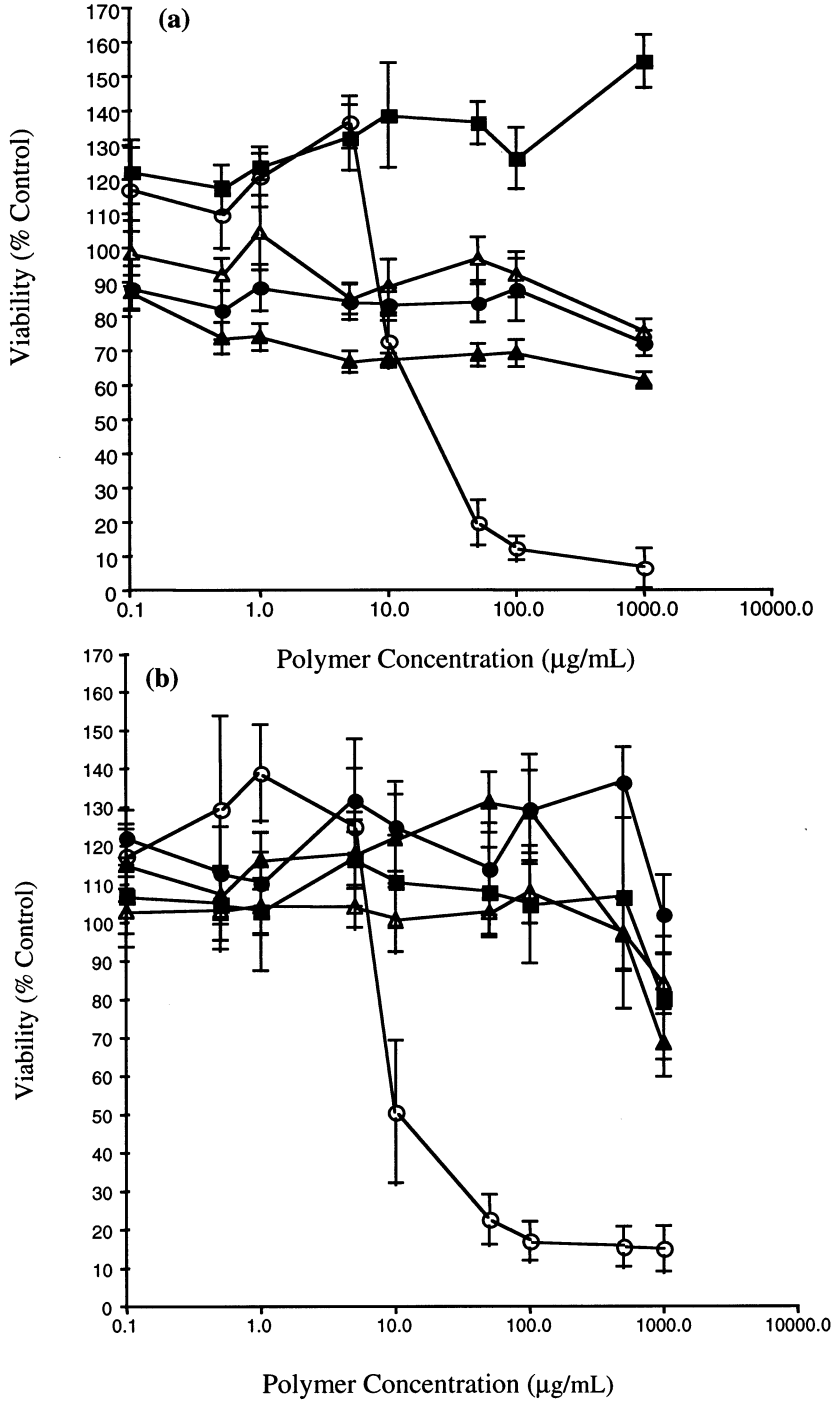

Fig. 2. Cytotoxicity of chitosan fractions in vitro. (a) L132 cells, (b) CCRF-CEM cells. The cytotoxicity of chitosan fractions N1 (▲), N2 (●) and N3 (■) and of the reference controls dextran (△) and poly(L-lysine) (○) is shown. Values are mean ± S.D. ($n = 18$).

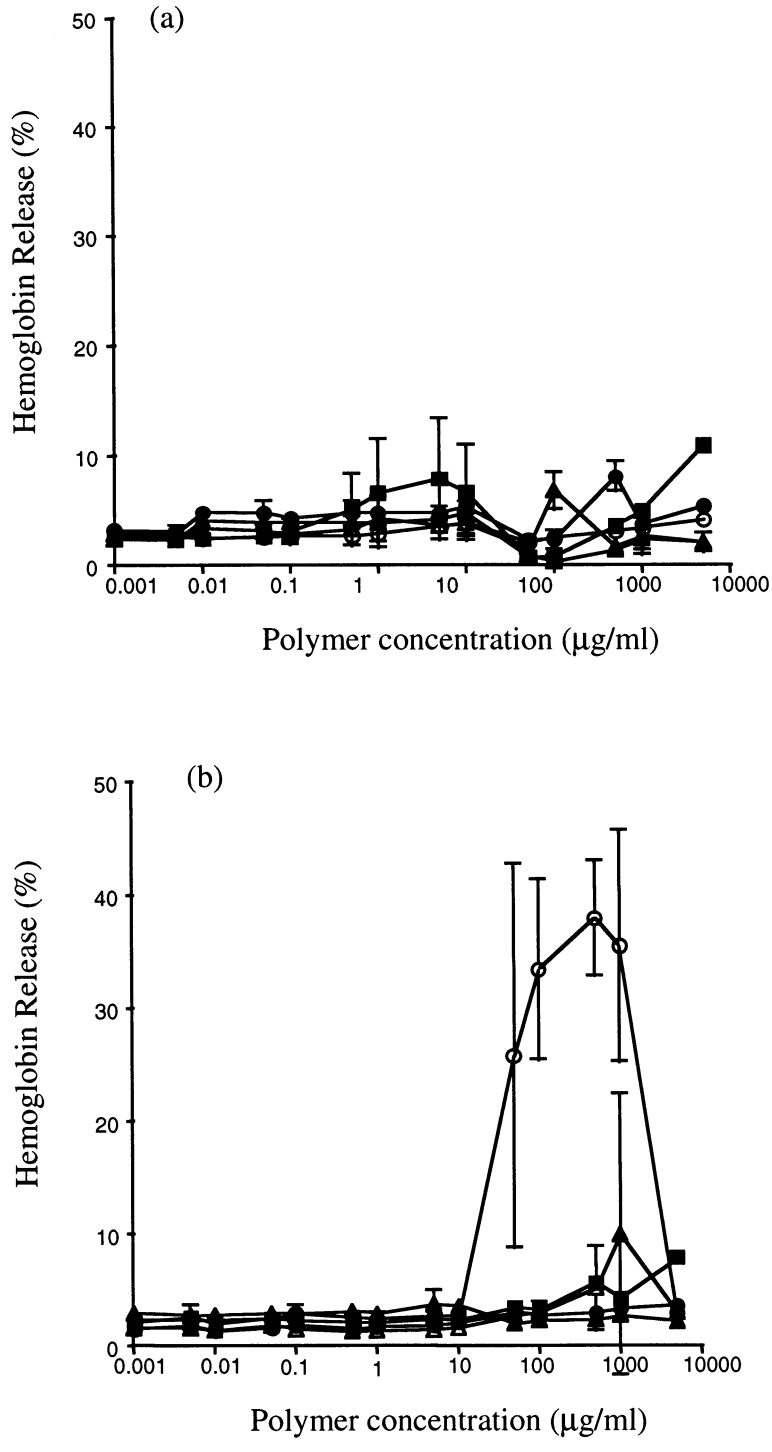

Fig. 3. Effect of chitosans on red blood cell lysis at (a) 1 h, (b) 5 h. The effects of N1 (▲), N2 (●) and N3 (■) and of the reference controls dextran (△) and poly(L-lysine) (○) are shown. Values are mean ± S.D. ($n = 12$).

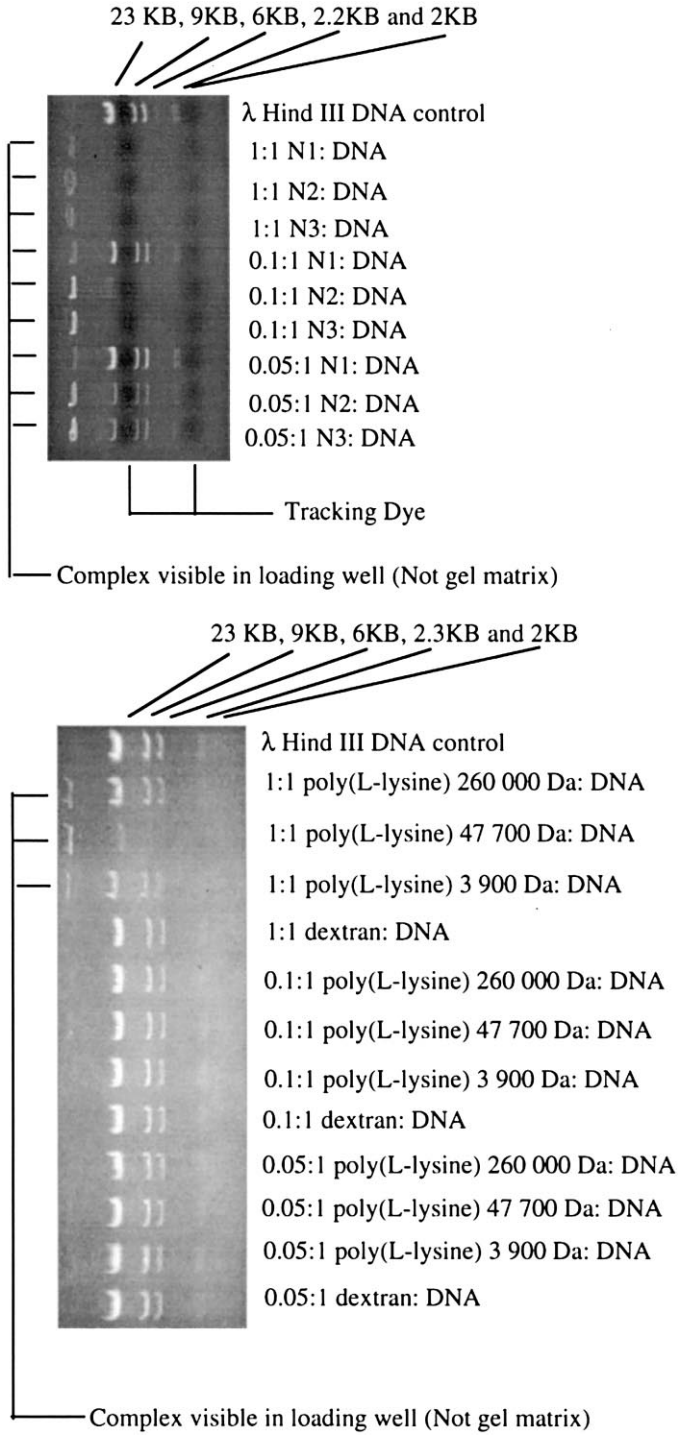

Fig. 4. Agarose gel electrophoresis of polymer–DNA complexes. Polymer was mixed with a λ *Hin*dIII DNA digest (standard molecular weight ladder) in the charge and weight ratios shown (see Section 2) (a) Chitosan complexes, (b) dextran and poly(L-lysine) complexes.

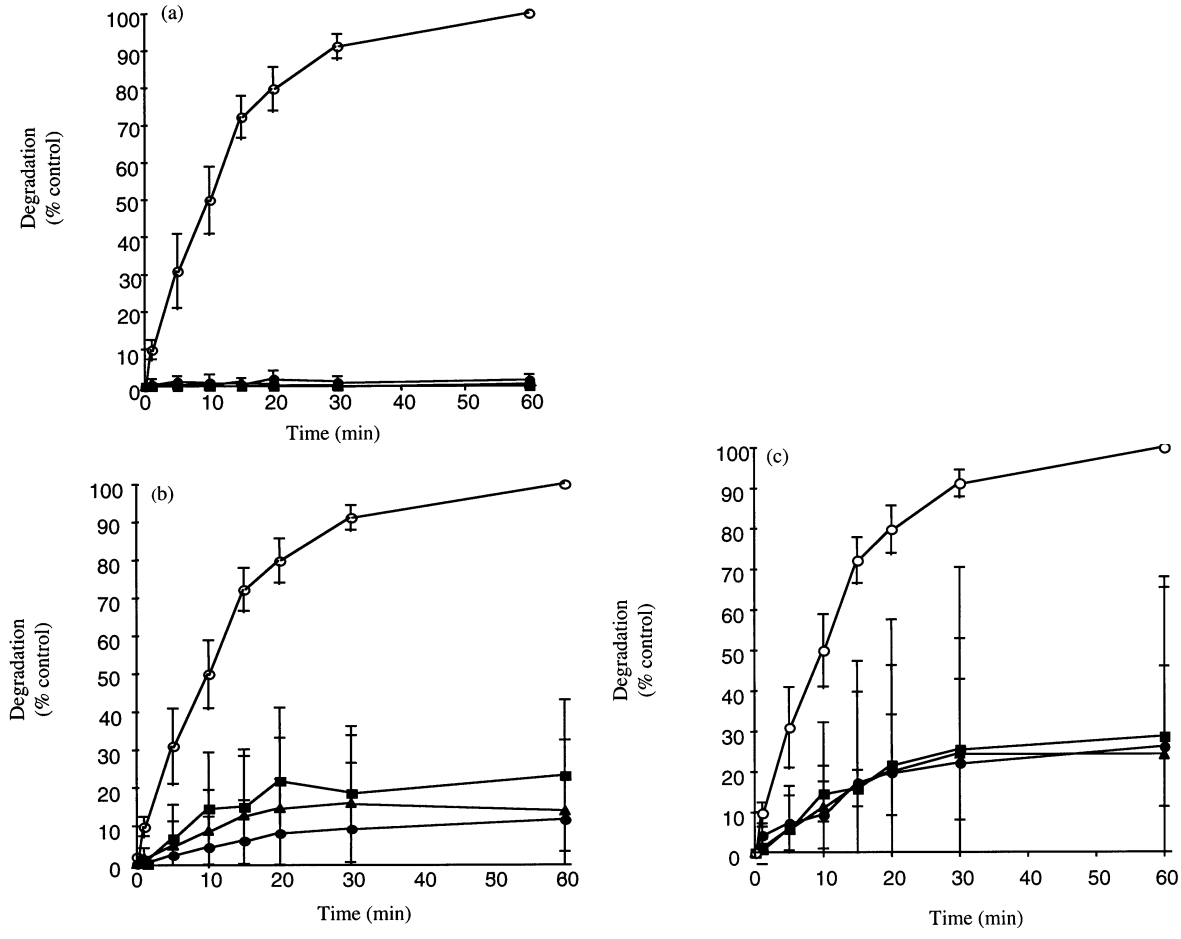

Fig. 5. Effect of polymer–DNA complexation on degradation by DNase II. Degradation of DNA (○) and the chitosan–DNA complexes formed from N1 (■), N2 (▲) and N3 (●) is shown. (a) Charge ratio of 1:1, (b) charge ratio of 1:0.1, (c) charge ratio of 1:0.05. Values are mean ± S.D. ($n = 3$).

gamma camera imaging should elucidate the mechanism of blood clearance. The hepatotropism of the higher molecular weight chitosans N2 and N3 does not bode well for their use in DNA delivery systems designed for targeting to other tissues. The distribution of chitosan poly-

Table 2
Recovery of dose administered after intravenous injection of ^{125}I-labelled polymers

^{125}I-labelled polymer	Recovery[a] of dose administered at 5 min after injection (mean ± S.D.)	Recovery[a] of dose administered at 60 min after injection (mean ± S.D.)
HPMA copolymer-$TyrNH_2$	83.1 ± 4.1	56.0 ± 5.8
N1	16.1 ± 8.0	31.8 ± 3.8
N2	46.0 ± 6.8	55.8 ± 19.2
N3	36.4 ± 18.5	47.1 ± 11.6

[a] Total radioactivity recovered in the organs sampled expressed as a percentage of administered dose.

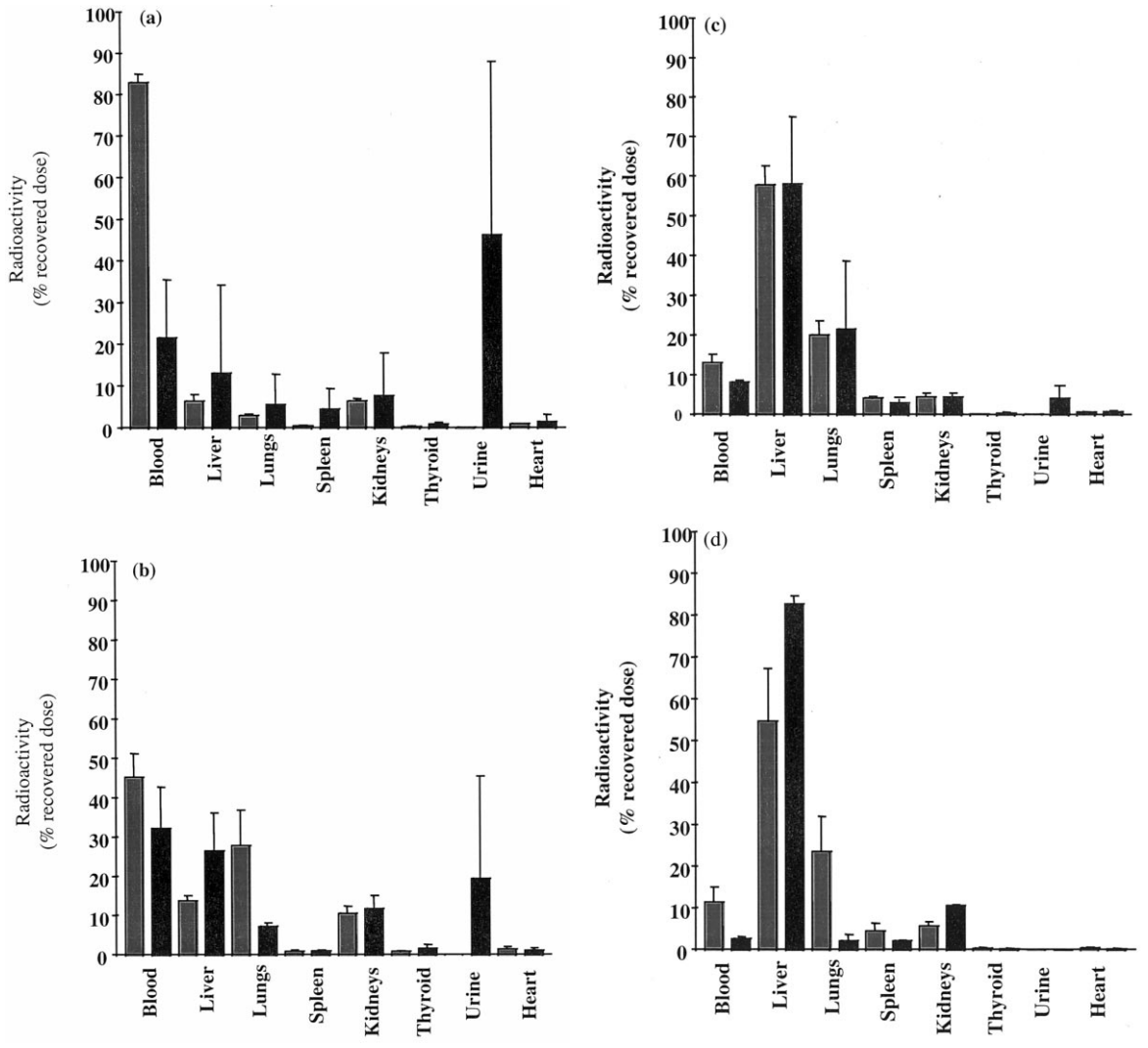

Fig. 6. Body distribution of ^{125}I-labelled chitosans after intravenous administration to rats. (a) Body distribution of ^{125}I-labelled HPMA copolymer-TyrNH_2, as control. (b) ^{125}I-labelled N1. (c) ^{125}I-labelled N2. (d) ^{125}I-labelled N3. Body distribution at 5 min (▩) and 60 min (■) is shown. All results are expressed as percentage recovered dose (mean ± S.D., $n = 3$).

plexes remains to be studied. In contrast, we have shown that polyamidoamines (linear polymers having amido and tertiary amino groups along the polymer backbone; Ferruti et al., 1998) can be designed that complex DNA and, unlike chitosans of similar molecular weight, do not show liver uptake after intravenous administration (Richardson et al., 1998)

4. Conclusions

Purified chitosan fractions can be prepared that are neither haemolytic nor cytotoxic, and these materials have potential for use as parenteral drug or DNA carriers. These low molecular mass chitosans complex DNA more effectively than poly(L-lysine) does and protect against nuclease

degradation. After intravenous administration, ^{125}I-labelled chitosans were cleared relatively quickly. In the case of ^{125}I-labelled chitosan of molecular mass >5000 Da, significant liver uptake was observed; this observation may call into question its potential usefulness in systems designed for targeting tissues other than liver. However, chitosan of <5000 Da was not hepatotropic.

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