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Preparation and evaluation of nanoparticles made of chitosan or *N*-trimethyl chitosan and a cisplatin–alginate complex

S. Cafaggi [a,*], E. Russo [a], R. Stefani [a], R. Leardi [a], G. Caviglioli [a], B. Parodi [a], G. Bignardi [a], D. De Totero [b], C. Aiello [b], M. Viale [b]

[a] *Dipartimento di Chimica e Tecnologie Farmaceutiche ed Alimentari, via Brigata Salerno 13, 16147 Genova, Italy*
[b] *Istituto Nazionale per la Ricerca sul Cancro, S.C. Terapia Immunologica-Genova, Italy*

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Abstract

In this work, nanoparticles with a negative or positive surface charge were prepared through electrostatic interaction of an anionic cisplatin–alginate complex with a cationic polyelectrolyte, namely chitosan or *N*-trimethyl chitosan (substitution degree of 85%). Statistical experimental design allowed the study of the influence of component amounts on the characteristics of nanoparticles. Mean particle diameter ranged from 180 nm to 350 nm. After 24 h, while the cisplatin–alginate complex released almost all the drug in saline-buffered solution at pH 7.4, approximately 40% w/w of total cisplatin was released from negative nanoparticles and roughly 50% w/w from positive ones. The same cumulative amounts of released drug were found after 48 h, with a progressive reduction to lower values up to 6 days. Drug loading of nanoparticles with a positive zeta potential (43 mV–60 mV) ranged from 13% w/w to 21% w/w and particle yield, referred to total polymers, was about 15% w/w (50% w/w if referred to cisplatin–alginate complex). Nanoparticles with a negative zeta potential (−34 mV) were obtained with a yield of 40% w/w and a drug loading of 18% w/w. These nanoparticles were the least active on all cell lines tested, while the cytotoxic activity of the positive nanoparticles was similar to or lower than that of cisplatin, probably depending on the combination of sizes and zeta potential values, on P388 murine and A2780 human cells. On A549 human cells, the nanoparticles with the smallest size and the lowest positive zeta potential were more active than cisplatin and showed a similar capability in inducing apoptosis in A2780 human cells. These results indicate that cisplatin complexes with polycarboxylate polymers can be transformed into cisplatin particulate carriers of high potential interest.

Keywords: Nanoparticles; Alginate; Quaternized chitosan; Cisplatin; Cytotoxic activity

1. Introduction

Cisplatin is a widely used antineoplastic drug that exhibits therapeutic activity against several solid tumors [1,2]. Nevertheless its high antitumor activity is frequently hampered by relevant side effects, in particular nephrotoxicity, neurotoxicity and ototoxicity [3]. Great efforts have been made to reduce these effects by means of biological and pharmacological strategies also aiming to permit higher dosages of drug, which could be of particular benefit from a therapeutic point of view [4]. A more selective drug delivery was achieved using water soluble drug–polymer conjugates, such as complexes with poly-(amidoamines) [5], poly(amidoamine) dendrimers [6] and *N*-(2-hydroxypropyl) methacrylamide (HPMA) [7]. Among the polymeric drug systems, complexes between cisplatin and polysaccharides containing carboxylic groups, such as hyaluronic acid, poly-L-aspartic acid, carboxymethyl dextran and poly-L-glutamic acid, are of particular interest [8]. The latter two complexes showed antitumor activity against human ovarian carcinoma cells cultured *in vitro* and therapeutic activity *in vivo* following intraperitoneal treatment of xenografted ascitic ovarian tumor in nude mice. More recently, the *in vivo* activity of a complex between cisplatin and poly-γ-glutamic acid was found to be superior to cisplatin, indicating that such a conjugate may be an effective carrier for the drug and may prove useful in the treatment of human breast cancer [9]. Complexes of

* Corresponding author. Tel.: +39 10 353 2625; fax: +39 10 353 2684.
E-mail address: cafaggi@dictfa.unige.it (S. Cafaggi).

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cisplatin with alginates [10], which belong to the above category of carriers, inhibited accumulation of Pt in the kidneys and favourably altered the drug's pharmacokinetics and nephrotoxicity, while maintaining its *in vitro* antitumor activity. Among particulate systems, thermosensitive liposomes [11], pegylated liposomes [12] and polymeric micelles based on poly(ethylene glycol)–poly (aspartic acid) copolymers [13] were used to improve tumor targeting, through a prolonged blood residence achieved by these cisplatin carriers after i.v. administration. Long-circulating PLGA-mPEG nanoparticles loaded with cisplatin were recently prepared using a modified double emulsion method. The composition (PLGA:mPEG ratio) of these nanoparticles appeared to affect the blood profile of the drug, which, from a pharmacokinetics standpoint, constituted an improvement with respect of the results achieved with the cisplatin–alginate complex mentioned above [14]. Nanoparticles useful for sustained drug release can be also obtained by electrostatic interaction between alginate and chitosan [15]. Both alginate and chitosan have been widely used in drug delivery [16]. Chitosan is a natural cationic polysaccharide derived by deacetylation of chitin, a copolymer consisting of combined units of glucosamine and *N*-acetyl glucosamine [17,18]. In the pharmaceutical field chitosan's advantageous biological properties have prompted its extensive study as a carrier both of drugs [19,20] and of proteins [21]. Oral administration of cisplatin in the presence of chitosan in sarcoma 180-bearing mice was shown to be useful for the prevention of body weight reduction and immunogenicity induced by the drug, without loss of antitumor activity [22]. In addition to its ability to facilitate paracellular transport of peptides and proteins across mucosal barriers [23], chitosan is biodegradable and has a very low toxicity. It was found that the addition of chitosan increases the mechanical strength of alginate microparticles [24]. A major drawback of chitosan is that it is insoluble at physiological pH, whereas it is soluble and active as an absorption enhancer only in its protonated form in acidic environments [25]. By contrast, *N*-trimethyl chitosan chloride (TMC), a chitosan derivative, obtainable at different degrees of quaternization, has a good water solubility over a wide pH range. Moreover, soluble TMC has mucoadhesive properties and an absorption enhancing effect even at neutral pH [26]. There are some reports about the toxicity of TMC [27,28], but only limited toxicity data have been published for particulate TMC systems [29,30]. Sodium alginate is an anionic polysaccharide obtained from marine algae. It consists of D-mannuronate and L-guluronate residues, which are arranged in both homopolymeric and heteropolymeric blocks. Alginate has been used in pharmaceutical formulations as an antiulcer agent, antacid agent and wound protectant. Nanoparticles made of alginate and polylysine were described [31] and later prepared for antisense oligonucleotides delivery [32]. Calcium ion is generally used to obtain, from alginate macromolecules, egg-box like structures [33], that result from the interaction between the ion and the carboxylate units of the guluronic residues (ionotropic gelation) [34]. The subsequent addition of a polyelectrolyte bearing an opposite charge with respect to alginate makes it possible to obtain particles of various sizes, depending on several factors. Studies have been performed to discern the optimal conditions for the formation of nanoparticles. It was found that an alginate concentration of 0.5 mg/mL in the final system represents a good starting point. Calcium ion and polyelectrolyte concentrations must be within a determined range in order to prevent aggregate instead of nanoparticle formation [15,31]. More recently, Douglas and Tabrizian [35] showed that, using the same procedure described above to prepare nanoparticles by interaction of chitosan with alginate, the solution pH must be controlled in order to avoid aggregate formation at higher chitosan concentrations. On the other hand, Lin et al., in preparing nanoparticles composed of a soluble chitosan and poly-γ-glutamic acid, introduced a procedure involving inverted addition of the components, using solutions at a proper concentration and at a controlled pH value [36].

The approach investigated in the current work entailed starting from suitable complexes between cisplatin and polycarboxylate polymers, which have shown their promise as cisplatin carriers [10,37,38], to obtain nanoparticulate systems. Such a transformation could impart advantageous new properties to these systems, since it allows to calibrate their sizes within the nanometre range, to modulate the particle surface charge, rendering it negative or positive depending on the polyelectrolyte weight ratios employed, and to achieve a better control of drug release. These features could very well play an important role in modifying the *in vitro* and *in vivo* antitumor activity and toxicity of the conjugated drug. In fact, for the delivery to solid tumors *in vivo*, nanoparticles are preferentially distributed in the vasculature due to the enhanced permeability and retention (EPR) effect [39]. It has been shown that the pore size of most peripheral human tumors ranges from 200 nm to 600 nm in diameter, with a mean of about 400 nm [40]. Moreover, nanoparticles with a positive surface charge are preferentially taken up by the tumor and retained for a longer time span compared to negatively charged or neutral particles [41].

The aim of this work was to prepare nanoparticles consisting of alginate, previously complexed with cisplatin, and chitosan or TMC, this latter being used when positively charged particles were desired. Statistical experimental design was employed to evaluate the influence of the component concentrations on particle size and zeta potential and to identify suitable formulations for subsequent study. Drug release capability *in vitro* was also investigated. Results of experiments on several tumor cell lines performed to evaluate the nanoparticles' cytotoxic activity *in vitro* and their ability to induce apoptosis, are also presented.

2. Materials and methods

2.1. Materials

Chitosan and alginic acid sodium salt were purchased from Sigma Aldrich (Milwaukee, USA). Chitosan was of low molecular weight (50,000–190,000), with a degree of deacetylation of 85%, 53 mPa s viscosity at $C=1\%$ w/w in 1% w/w acetic acid aqueous solution, at 25 °C. Alginic acid sodium salt, composed of 39% guluronic acid and 61% mannuronic acid, was of low molecular weight (12,000–80,000); the viscosity of an aqueous solution ($C=2\%$ w/w) was approximately 250 mPa s, at

25 °C. Cisplatin and tin(II) chloride dihydrate ($SnCl_2 \cdot 2H_2O$) were also purchased from Sigma Aldrich. All the other chemicals were of reagent grade. Water was purified by Milli-Q Plus system (Millipore, USA).

2.2. Methods

2.2.1. Preparation of cisplatin–alginate complex

The cisplatin–alginate complex was obtained using the procedure described in literature [11], with some modifications regarding alginate concentration, cisplatin to alginate weight ratio and experimental conditions. The concentration of sodium alginate was fixed at 0.600 mg/mL, whereas that of cisplatin varied from 0.050 mg/mL to 0.250 mg/mL, in order to achieve different drug to uronic acid molar ratios (in the range from 0.050 to 0.27). Cisplatin and sodium alginate were dissolved in purified water and the solution was magnetically stirred in the dark for 24 h in a water bath set at 40 °C ± 5 °C. Thereafter, the complex solution was stored in the refrigerator until use.

2.2.2. Evaluation of the extent of reaction between cisplatin and alginate

The extent of reaction was initially determined using the method proposed by Nishiyama et al. [42] with slight modifications. Fifteen milliliters of the reaction mixture was dialyzed to equilibrium against 400 mL of distilled water using a dialysis membrane (Spectra/Por-7; MWCO: 1000, Spectrum Laboratories, Inc., Laguna Hills, CA). Twenty four hours later, 50.0 mL of dialysate was sampled, concentrated to 5.00 mL and 1.00 mL was mixed with 2.00 mL of 4 M HCl–0.4 M $SnCl_2$ solution. The cisplatin content in the solution was determined spectrophotometrically, after 15 min, based on the absorbance at 403 nm of the Pt–$SnCl_2$ complex formed. From the cisplatin content in the dialysate thus determined, the amount of cisplatin reacted with the polymer was calculated, on the basis of a calibration curve obtained from standard solutions of cisplatin in water. The calibration curve was linear in the concentration range 2 μg/mL–30 μg/mL, with an intercept value not statistically different from zero, R^2 (correlation coefficient) > 0.99. A parallel experiment was performed to evaluate the adsorption of cisplatin onto the membrane. Fifteen milliliters of a cisplatin solution at a concentration of 21.0 μg/mL was dialyzed as described above for the reaction mixture and the cisplatin released after 24 h was determined by the same reactive.

Experiments were performed in triplicate.

The extent of reaction was also evaluated using two alternative methods. The first of the two was derived from the method described above. Salts were added to 3.0 mL of the reaction mixture in order to obtain the composition of phosphate buffered saline (PBS) (10 mM phosphate buffer at pH 7.4, 150 mM NaCl) employed as the receptor phase (V = 60 mL) in a dialysis process carried out under stirring at room temperature. At pre-determined time intervals (15, 30, 60, 120, 180 and 300 min) aliquots (V = 3.0 mL) of the receptor phase were withdrawn and immediately replaced by an equal volume of fresh buffer. An appropriate volume of each sample was mixed with an equal volume of a 4 M HCl–0.4 M $SnCl_2$ solution. After 15 min, the released cisplatin was determined spectrophotometrically, measuring the absorbance at 403 nm of the Pt–$SnCl_2$ complex formed, by means of a calibration curve based on standard solutions of cisplatin in PBS (concentration range 2 μg/mL–20 μg/mL, R^2 = 0.997 with an intercept value not statistically different from zero). The amount of cisplatin reacting with the polymer was then calculated from the intercept of the linear part of the release curve, considering that the relative amount of drug released from the complex at time 0 corresponded to the free cisplatin present in the reaction mixture.

The second method was based on size-exclusion chromatography. Salts were added to an aliquot of the reaction mixture in order to obtain the composition of PBS and 20 μL of the resulting solution was injected, every 18 min for up to 5 h, into a 300 mm × 7.8 mm TSK-GEL® G5000PW_{XL} column (Tosoh Bioscience, Stuttgart, Germany) eluted with water at a flow rate of 1.0 mL/min. The HPLC operation was performed at room temperature using an HP1090 series II liquid chromatograph equipped with a diode array detector (DAD) managed by an HPLC Chem Station (DOS series) from Hewlett Packard. The analytical DAD wavelength was set at 206 nm. The free cisplatin present in the reaction mixture was calculated from the intercept of the linear part of the plot of cisplatin concentration versus time, following reference to a calibration curve based on standard solutions of cisplatin in PBS (concentration range 2 μg/mL–20 μg/mL, R^2 = 0.998 with zero intercept). The retention time of free cisplatin was 15.3 min.

2.2.3. Synthesis of TMC

N-Trimethyl chitosan chloride was synthesized by methylation of chitosan with CH_3I in the presence of a strong base (NaOH), following the procedure proposed by Polnok et al. [43], except for product purification. A first reaction step was performed, followed by two addition steps, in order to achieve a high degree of quaternization. In detail, a mixture of 2 g of chitosan, 4.8 g of sodium iodide and 10 mL of 20% (w/w) aqueous NaOH in 80 mL *N*-methylpyrrolidinone was maintained in a water bath under stirring at 60 °C for 20 min in the presence of a Liebig condenser. Then, 12 mL CH_3I was added to the mixture and the reaction was carried out for another 60 min. At this point the first addition step was realized by adding 5 mL of CH_3I and 10 mL of 20% (w/w) NaOH and continuing the reaction for 60 min at the same temperature. After this time a second addition step, equivalent to the first, was performed and the reaction was stopped after 60 min.

The product was then precipitated by adding ethanol and diethyl ether, and after washing on a glass filter with diethyl ether, it was collected and dried on silica gel in a dessicator.

The dried product was then dissolved in a 5% (w/w) NaCl aqueous solution (15 mL for a portion of 200 mg product) and maintained under stirring for 1 h to exchange iodide ions with chloride ions. Afterwards, the solution was purified by dialysis against water until no precipitation of chloride ions was detected by reaction of an aliquot of dialysis medium with silver nitrate in the presence of diluted nitric acid. The final product was obtained in the dried state by freeze-drying (yield of about 15% w/w, calculated as the ratio between the weight of purified trimethylated product and the weight of the starting chitosan).

2.2.4. Characterization of TMC

The purified TMC was analyzed by ^{1}H-nuclear magnetic resonance spectroscopy (NMR). The ^{1}H-NMR spectrum of the TMC in D_2O was recorded with an NMR spectrometer (AV-300, Brucker, Switzerland). The degree of quaternization (DQ %) was calculated using the following equation [40]:

$$DQ = [(CH_3)_3]/[H] \times 1/9 \times 100$$

where $[(CH_3)_3]$ is the integral of the hydrogens of the trimethyl amino group at 3.3 ppm and [H] is the integral of the hydrogen peaks between 4.7 and 5.7 ppm.

The degrees of substitution at the 3- and 6-hydroxyl groups of chitosan were calculated from the same ^{1}H-NMR spectrum. The chemical shifts of 3- and 6-hydroxymethyl groups were at 3.5 and 3.4 ppm, respectively. The degree of *O*-methylation (DOM %) of the polymer was calculated using the following equation:

$$DOM = [(CH_3)_3]/[H] \times 1/3 \times 100$$

where $[(CH_3)_3]$ is the integral of the hydrogens of the hydroxymethyl group.

2.2.5. Preparation of nanoparticles

2.2.5.1. Procedure A. A chitosan solution (C=0.500 mg/mL) in 3% v/v acetic acid was prepared and its pH adjusted to 5.5 by 6 M NaOH. Different volumes of this solution were added to a fixed volume (10.0 mL) of cisplatin–alginate complex solution ($C_{alginate}$=0.600 mg/mL), in order to obtain samples containing nanoparticles of different composition in terms of chitosan to alginate weight ratio. Chitosan solution was added at a controlled flow rate (0.5 mL/min) by a peristaltic pump with the terminal Teflon tube tip immersed into the liquid in order to avoid droplet formation, thereby ensuring a slow delivery without the formation of zones at higher chitosan concentration. Adding was performed under magnetic stirring (400 rpm) maintained for 30 min. All samples were diluted with water, if necessary, to the same final volume (14.0 mL). The final alginate concentration was C=0.429 mg/mL. Measurements of particle diameter were made, unless otherwise specified, on the prepared sample after dilution, without preliminary separation of nanoparticles and subsequent reconstitution of the sample.

2.2.5.2. Procedure B. Appropriate quantities of cisplatin–alginate complex were added to an aqueous solution (5.0 mL) containing different amounts of TMC (ranging between 1.34 mg and 5.67 mg). The complex amounts, calculated as sodium alginate, ranged from 0.60 mg to 1.05 mg. Adding was performed by delivering, at a controlled flow rate as described for procedure A, appropriate volumes (from 1.0 mL to 1.75 mL) of the cisplatin–alginate complex solution. The cisplatin–uronic acid molar ratio was fixed at 0.216, corresponding to a cisplatin to alginate weight ratio of 0.33. Samples were then diluted, when necessary, with water to the final volume (7.0 mL) and maintained under magnetic stirring at room temperature for 30 min. Measurements of particle size were made as indicated in procedure A.

2.2.5.3. Blank nanoparticles. Nanoparticles not loaded with drug were obtained by the same methods described above, starting from a sodium alginate solution at the same concentration used in the preparation of the cisplatin–alginate complex, subjected to heating for 24 h in a water bath set at 40 °C±5 °C, as for the reaction with cisplatin.

2.2.6. Characterization of nanoparticles

Particle size (average apparent diameter, D) and polydispersity index (PDI) were determined by dynamic light scattering using a photon correlation spectroscopy (PCS) assembly (Zetasizer 3000 HS, Malvern Instruments, UK). Determinations were carried out at 25 °C at a fixed angle of 90°. Samples were diluted with two volumes of 1 mM NaCl solution before measurement. Results are reported as the mean of five measurements±SD (standard deviation).

The zeta potential (Z) was measured by laser Doppler anemometry on the same instrument. Measurements were made at 25 °C without sample dilution or any salt addition. Results are reported as the mean of ten measurements±SD.

The shape and surface characteristics of the nanoparticles were observed by scanning electron microscopy (SEM). The nanoparticle sample was thinly sprinkled onto a metal stub and vacuum coated with a thin layer of gold in an argon atmosphere. The coated samples were examined at an acceleration voltage of 20 kV (Steroscan 440, Leica Cambridge, Ltd., Cambridge, UK).

Purification of nanoparticles was achieved by centrifugation (16,000×g for 30 min at T=10 °C) on a glucose (33% w/w) bed (50 μL) and redispersion in water.

2.2.7. Statistical experimental design

Doehlert designs for two variables were used to study the influence of amounts of chitosan or TMC and different cisplatin–uronic acid molar ratios or quantities of cisplatin–alginate in the sample on the D and Z of nanoparticles by the response surface methodology [44]. Designs of experiments and data analysis were performed using Modde software, Version 6.0 (Umetrics, Sweden) and Matlab routines written by the authors. Statistical significance was set at $p<0.05$.

2.2.8. Drug loading, nanoparticle yield and yield of transformation

Cisplatin content in nanoparticles to be used for the drug release study was determined routinely by an indirect procedure: this approach was adopted because the method of analysis was destructive and would have involved loss of sample. The nanoparticles were centrifuged as described in Section 2.2.6. and the drug in the supernatant was quantified by the tin(II) chloride method, as described in Section 2.2.2. Determination was carried out using cisplatin standard solutions containing an equal amount of polymer present in excess in the formulation, in order to compensate for the matrix effect that would have otherwise negatively influenced measurements. Cisplatin present in nanoparticles was estimated by subtracting the fraction of cisplatin remaining in the supernatant from the total amount of cisplatin present in the suspension before centrifugation. Inductively coupled plasma atomic emission

spectroscopy (ICP-AES) was used to check the results obtained by the colorimetric method. Measurements were performed at $\lambda = 224.552$ nm on a J.Y. 24 spectrometer (Jobin-Yvon, Longjumeau, France), equipped with a Cetac U-5000AT+ ultrasound nebuliser (Cetac Technologies Inc., Omaha, Nebraska, USA). Calibration curves were linear in the concentration range (10–60) μg/mL ($R^2 > 0.99$).

To verify the accuracy of the indirect determination of cisplatin content, some samples were also evaluated by a direct procedure, which allowed to use small amounts of sample, though it was time-consuming. This entailed evaporating an accurately measured aliquot of a purified nanoparticle suspension (0.100 μL) and digesting it in concentrated nitric acid (1 mL) at a temperature of about 120 °C for 24 h, in order to eliminate the organic substance. Nitric acid was then evaporated and substituted with 1 mL of *aqua regia* to ensure the complete solubilisation of any residual Pt. After 1.5 h at a temperature of about 80 °C, *aqua regia* was evaporated, and the dried residue was dissolved in an appropriate volume of the 4 M HCl–0.4 M $SnCl_2$ solution and diluted with an equal volume of water. Spectrophotometric determination of cisplatin was then carried out by measuring the absorbance of the Pt–$SnCl_2$ complex formed as described in Section 2.2.2, on the basis of a calibration curve obtained from aqueous standard solutions of cisplatin (concentration range 10 μg/mL–80 μg/mL, $R^2 > 0.99$). Results from the direct and the indirect methods did not differ by more than ±15%.

Nanoparticles samples were lyophilized after centrifugation in the absence of glucose bed and weighed to determine drug loading. This was calculated as the percent ratio between the cisplatin amount determined by the indirect assay and particle weight. Nanoparticle yield was calculated as the percent ratio between the particle weight and total component amounts used in the sample preparation. The relative amount of complex undergoing the transformation into nanoparticles (yield of transformation) was calculated as the percent ratio between the complex amount in the particles and the starting complex weight. Taking into account the extent of complexation reaction and knowing the cisplatin to alginate weight ratio in the reaction mixture, the amount of complex in nanoparticles was derived from the amount of the contained cisplatin, as determined by the indirect procedure after centrifugation of the sample.

2.2.9. Drug release study in vitro

The release of cisplatin was evaluated by the dialysis method. Salts were added to the solution of cisplatin–alginate complex in order to obtain the composition of the PBS used as the receptor phase. A cisplatin solution in the same medium was prepared for a control experiment ($C = 0.200$ mg/mL). Dialysis bags containing 3.0 mL of the free drug or complex solution in PBS were immersed in a thermostated beaker containing 60.0 mL PBS at 37.0 °C±0.1 °C and maintained under agitation with a stirring bar rotating at 300 rpm. At predefined intervals, aliquots ($V = 3.0$ mL) of receiving buffer were withdrawn and cisplatin content was determined by reaction with $SnCl_2$, on the basis of the calibration curve obtained from standard solutions of cisplatin in PBS. Each aliquot was immediately replaced by an equal volume of fresh buffer. In the case of nanoparticles, they were recovered from the initial suspension ($V = 28$ mL) by centrifugation as described in Section 2.2.6. and redispersed in 1.5 mL PBS. This was then transferred into the dialysis bag for the release study that was carried out using the same procedure described above. The initial cisplatin content in nanoparticles was determined by the indirect procedure described in the previous section.

2.2.10. Growth inhibition assay

Human A2780 (ovary, carcinoma) and A549 (lung, carcinoma) cells, and murine P388 (leukaemia) cells were plated at different concentrations/well (P388: 1500 cells/well; A549: 1600 cells/well; A2780: 2000 cells/well) into 96-well microtiter plates (flat-bottomed for adherent cells and U-bottomed for non-adherent cells) for about 8 h. Cisplatin, nanoparticles after centrifugation and redispersion in water, and cisplatin–alginate complex were then added at the appropriate concentrations for a minimum of 5 concentrations (2-fold serial dilutions). Cells were treated in duplicate and a final volume of 200 μL was reached in each well. After 3 days culture, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) solution (2 mg/mL in PBS) was added to the wells and incubated at 37 °C for 4 h. Microplates were then centrifuged at 275×*g* for 5 min and the culture medium carefully aspirated and replaced with 100 μL of 100% dimethylsulfoxide. Complete and homogeneous solubilisation of formazan crystals was achieved after 20 min of incubation and a slight plate shaking. The absorbance was measured on a 400 ATC microculture plate reader (SLT Labinstruments, Austria) at 540 nm. IC_{50}s were calculated by the analysis of single dose response curves, each final value being the mean of 8–9 independent experiments [45].

2.2.11. Apoptosis assay

The induction of apoptosis was evaluated in A2780 cells treated with cisplatin, cisplatin–alginate complex and nanoparticles after centrifugation and redispersion in water. Cells were plated into 75 cm^2 flasks and treated with their specific IC_{50} for each compound. Floating and adherent cells were harvested after 24 h and 72 h culture, washed twice with cold PBS containing 5% v/v fetal calf serum and apoptosis determined by double staining with Annexin-V-FITC (Ann-V) and Propidium Iodide (PI) (rh Annexin-V-FITC Kit, Bender MedSystem GmbH, Vienna, Austria). Briefly, 0.5×10^6 cells were resuspended in binding buffer 1X and stained with 5 μL of Ann-V. After 10 min incubation 10 μL of PI was added and samples incubated for a further 5 min. Analysis was immediately performed using a Flow Cytometer (FACScan, BD Biosciences, Milano, Italy) with dedicated software.

3. Results and discussion

3.1. Cisplatin–alginate complex formation

Cisplatin was reacted with alginate at a fixed alginate concentration of 0.600 mg/mL, a value well below its critical

overlapping concentration, which was found to be about 4 mg/mL at 25 °C [46]. The chosen alginate concentration could directly allow the formation of egg-box like structures from alginate molecules in the presence of calcium ion, as described by Rajaonarivony et al. [29]. This could promote nanoparticle formation through the subsequent addition of chitosan, as reported by De and Robinson [15]. However, preliminary experiments (data not shown) indicated that calcium ion was not needed to obtain nanoparticles of suitable sizes, as already pointed out by Douglas and Tabrizian [35], who, moreover, proposed the control of the system's pH at about 5 in order to prevent particle aggregate formation when trying to use a greater chitosan–alginate weight ratio. Considering that the drug can lose two chloride ligands which can be replaced by carboxylates belonging to different chains, it was assumed that cisplatin might behave similarly to calcium ion in determining the formation of a compacted structure by interchain molecular crosslinking. At an alginate concentration of about 6 mg/mL used by Imai et al. [11], intermolecular interactions induced by cisplatin may be very pronounced due to chain overlapping. A situation of this kind had to be avoided, because such molecular aggregates, when massively present, could in turn determine the formation of nanoparticles of greater sizes, as indeed was observed in some preliminary experiments (not reported here). At the chosen dilution, interchain reactions between alginate and cisplatin might still occur, albeit to a reduced extent, also because the reaction needed to be performed at 40 °C in order to accelerate the interaction between the reagents and to prevent cisplatin degradation.

The reaction between cisplatin and alginate was found to be almost complete, at the different cisplatin to alginate molar ratios investigated here, with a yield of about 95% w/w (e.g., 93.0±2.0, by the chromatographic method and 94.7±2.6 by the modified dialysis method, both expressed as 95% confidence interval, for the complex at 0.216 cisplatin–uronic acid molar ratio). This value was similar to that reported by Imai et al. [11], who found a yield of about 90% (determined by preparative size-exclusion chromatography) after having carried out the reaction with 10-fold concentrated reagents at room temperature and using a fixed cisplatin–uronic acid molar ratio equal to 0.1. The method proposed by Nishiyama et al. [42], used here without modifications, could give inaccurate results because of the strong adsorption of cisplatin onto the membrane, as revealed by experiments carried out by dialyzing the free drug in water. In fact, the relative amount of cisplatin adsorbed from a solution at C=21.0 μg/mL was found to be (59.0±6.5)% w/w (expressed as 95% confidence interval). This was probably due to the low level of cisplatin concentration involved compared to that used by the cited authors. The yield of reaction calculated for the complex at 0.216 cisplatin:uronic acid molar ratio, taking into account the cisplatin adsorption, was equal to (94±10)% w/w (expressed as 95% confidence interval). Given the uncertainty of the result, two alternative methods were used in order to confirm it. Both of them resembled the known "standard additions" method employed in several analytical techniques when samples to be analyzed are based on a complex matrix. In the modified dialysis method, carried out at room temperature, the assumption was that if any free cisplatin was present in the reaction mixture, it would have been added to that derived from the dissociation of the cisplatin–alginate complex caused by the presence of PBS, which, on the other side, acted favourably by markedly reducing the cisplatin adsorption onto the membrane. The intercept of the linear part of the release curve, corresponding to time 0, would have given the desired amount. The result was expected to be independent of the experimental conditions, which should have influenced only the slope of the release curve. In fact, the obtained yield of reaction was consistent with what was found by considering the intercept of the linear part of the release curve registered for the cisplatin–alginate complex at 37 °C (see Section 3.5.) as the relative amount of drug released from the complex at time 0, corresponding to the free cisplatin present in the complex solution, was found to be (6.9±2.8)% w/w (expressed as 95% confidence interval). It is important to note that the reactive employed in the dialysis method was able to yield the yellow Pt–$SnCl_2$ complex with both cisplatin and its aquation products. Because the reaction was conducted in water, aquation products were certainly present if any free cisplatin remained in the reaction mixture. Analysis of cisplatin aqueous standard solutions by size-exclusion chromatography showed that aquation products gave a signal at about 4–8 min, coincident with that of the complex in the reaction mixture sample (data not shown). The signal of cisplatin at 15.3 min was due to the non-aquated product and appeared markedly reduced when an aqueous solution of cisplatin was subjected to the same thermal treatment as the possible free cisplatin in the reaction mixture. It was therefore necessary to transform aquation products into non-aquated cisplatin before considering its signal for quantisation. This was done by adding PBS to the reaction mixture, but, since PBS also caused the dissociation of the cisplatin–alginate complex, and this increased with time, the amount of free cisplatin present in the reaction mixture was obtained, in this case as well, from the intercept of the curve of cisplatin concentration versus time, on the basis of the same assumption above. It was found that adding PBS to cisplatin aqueous standard solutions caused the disappearance of aquation products after 1 h. According to this finding, the first points of the curve were not aligned with the subsequent ones, whereas linearity was again lost later as a consequence of the progressive establishment of the equilibrium between complexed and free cisplatin. An example of the obtained curves is represented in Fig. 1. Overall, the two alternative methods employed here confirmed the result of the unmodified dialysis method, but its uncertainty was undoubtedly reduced.

3.2. Preparation of nanoparticles made of chitosan and cisplatin–alginate complex

Negatively charged nanoparticles were prepared as indicated in procedure A, according to an initial Doehlert design for two variables, without replications, whose plan corresponding to the first seven runs is represented in Table 1. Such a design allowed investigating the influence on nanoparticle properties of different chitosan amounts and cisplatin–uronic acid molar

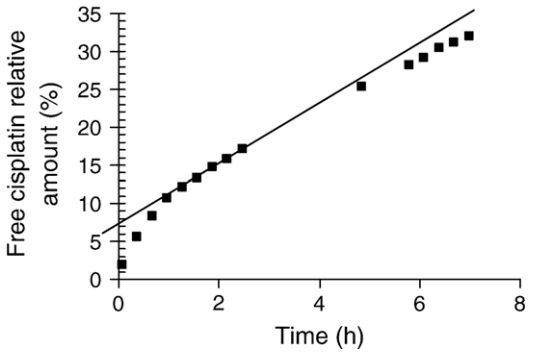

Fig. 1. An example of the curve of free cisplatin concentration versus time, obtained from HPLC measurements to determine the extent of reaction between cisplatin and sodium alginate.

ratios in the complex, taking into account that Imai et al. [10] used a fixed ratio equal to 0.10 (1:6 weight ratio of cisplatin–alginate).

The Doehlert design for two factors is a uniform shell design represented by a regular hexagon with a centre point. It allows extending or displacing the design in any of the six directions, overlapping with the original design and requiring only three extra experiments to be performed. The Doehlert design was used in order to find a suitable combination of variables that yielded nanoparticle samples characterized by low average diameter and narrow size distribution. The initial design indicated that an increase in the amount of cisplatin, and consequently in the cisplatin–uronic molar ratio, could lead to a decrease in nanoparticle size. The design was then extended in the direction which potentially allowed the generation of reduced particle size, taking into account that higher chitosan concentrations led to visible particle aggregates formation. Table 1 reports the experimental plan of the initial design, and the design extensions, which were performed in sequence, together with the results. In order to have a general description of the phenomena in the whole experimental domain under study, a global model taking into account the results of the 13 experiments was obtained, after having scaled the experimental conditions in the range −1/+1.

Having excluded run 6 as an outlier, the comprehensive experimental domain under study could be described for D by a refined quadratic model, based on the equation:

$$Y_1 = 339.7 + 3.5\ X_1 - 26.6\ X_2(***) - 38.4\ X_1X_2(**) - 22.2\ X_1^2(*)$$

$$R^2 = 0.86,\ Q^2 = 0.70, \text{RSD (residual standard deviation)} = 7.9\text{nm}$$

whereas, a further refined quadratic model was suitable for Z, for which no outlier was detected:

$$Y_2 = -34.5 + 2.2\ X_1 + 6.2\ X_2(***)$$

$$R^2 = 0.72,\ Q^2 = 0.66,\ \text{RSD} = 2.3\text{mV}$$

where Y_1 is the response variable D, Y_2 the response variable Z, X_1 the chitosan amount, X_2 the cisplatin amount (both to be introduced in the equations as coded values) and significant coefficients marked by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). R^2 is the fraction of variation of the response explained by the model, adjusted for degrees of freedom and Q^2 is the fraction of total variance explained in cross-validation. R^2 and Q^2 provide the best summary of the fit of the model. Values close to 1 for both parameters indicate a very good model with excellent predictive power. The response contour plots for D and Z are represented in Fig. 2. An increase in chitosan amount did not lead to a significant variation of Z values, which instead significantly increased by increasing the cisplatin amount; by contrast, it played a more prominent role in determining the variation of D values. In fact, in this case there was a significant quadratic term for chitosan amount and, more importantly, a significant interaction with the cisplatin amount. Limiting the observation to the area corresponding to the experimental domain (that bounded by the broken line in the figure) it can be seen that particle size was significantly reduced when cisplatin amount increased at higher values of chitosan amount.

The progressive reduction of particle size with increasing cisplatin amount reacted with alginate was in line with the initial hypothesis and can be explained by what is known about the interaction between alginate and divalent cations [47–49]. These latter bind preferentially with guluronic acid blocks of the alginate macromolecule due to their "zig-zag" structure that can more readily accommodate them. The alginates adopt an ordered solution conformation, through association of the poly-guluronic sequences in the presence of calcium or other divalent cations of similar size. This results in an alignment of two chain sections yielding an array of coordination sites, which can favour further interaction with divalent cations. Similar arrangements,

Table 1
The experimental plan of the initial Doehlert experimental design and the extensions subsequently performed

Chitosan amount (X_1)		Cisplatin amount (X_2)					
Real value (mg)	Coded value	Real value (mg)	Coded value	Cisplatin–uronic molar ratio	D (nm)	Z (mV)	PDI
3.8	0.333	2.0	−0.500	0.108	365±4	−41±1	0.52±0.05
5.1	1.000	2.0	−0.500	0.108	345±3	−37±2	0.41±0.08
4.5	0.692	3.0	0.000	0.162	338±4	−30±1	0.50±0.06
3.2	0.026	3.0	0.000	0.162	334±4	−33±1	0.40±0.03
2.5	−0.333	2.0	−0.500	0.108	332±3	−40±2	0.38±0.02
3.2	0.026	1.0	−1.000	0.054	415±3	−40±2	0.64±0.10
4.5	0.692	1.0	−1.000	0.054	389±4	−38±2	0.43±0.09
1.9	−0.641	3.0	0.000	0.162	337±3	−34±1	0.47±0.10
2.5	−0.333	4.0	0.500	0.216	329±2	−31±1	0.50±0.04
3.8	0.333	4.0	0.500	0.216	318±3	−29±2	0.38±0.06
1.2	−1.000	4.0	0.500	0.216	322±3	−34±1	0.40±0.02
1.9	−0.641	5.0	1.000	0.269	322±5	−30±1	0.33±0.05
3.2	0.026	5.0	1.000	0.269	314±3	−31±1	0.37±0.04

Coded values for the variables X_1 (chitosan amount) and X_2 (cisplatin amount), related to the global model, are reported close to their real values. Chitosan and cisplatin amounts refer to 12 mg sodium alginate. Initial $C_{alginate}$=0.600 mg/mL. Final $C_{alginate}$=0.429 mg/mL. Experimental data are represented as mean values ±SD.

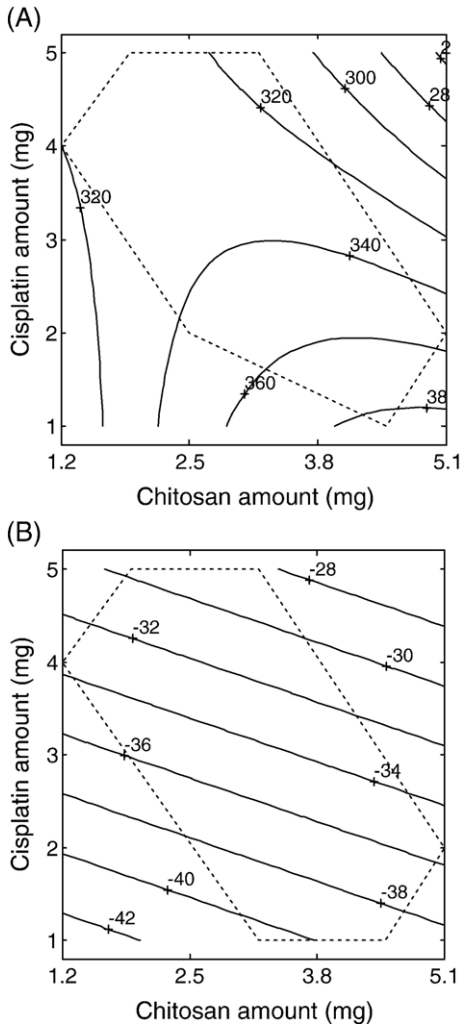

Fig. 2. Contour plots for D (nm) (A) and Z (mV) (B), over the entire experimental domain covered by the sequential Doehlert designs. The broken line defines the experimental domain.

not necessarily confined to guluronic moieties, were probably formed by Pt interchain crosslinking through coordination bonds with carboxylate anions. This chain interconnection could result in chain packing and, consequently, yield particles of reduced size after interaction with chitosan, the phenomenon becoming more evident in the presence of higher chitosan amounts.

Taking into account the experimental design results, the sample corresponding to the central point of the third design (2.5 mg chitosan and 4.0 mg cisplatin, corresponding to a 0.216 cisplatin–uronic acid molar ratio) was chosen for the subsequent investigation (NPS sample). Table 3 summarizes its experimental data.

3.3. Preparation of nanoparticles made of TMC and cisplatin–alginate complex

In order to obtain nanoparticles with a positive zeta potential, a chitosan derivative with a permanent positive charge (i.e., existing over a large pH interval), such as TMC with a high degree of quaternization, was used. This choice also took into account the risk that the presence of free amino groups in the chitosan possibly used in excess could, through the formation of irreversible coordination bonds with Pt, negatively interfere with the release of cisplatin from nanoparticles. On the other hand, a chitosan concentration suitable for positive nanoparticles was very difficult to achieve, due to its low solubility even in an acidic environment. TMC characterization by ^{1}H-NMR indicated that the degree of quaternization was 85%, according to the absence of the signals of mono- or dimethylated amino groups, whereas the degree of *O*-methylation at 3-and 6-hydroxyl groups was 64% and 83%, respectively. The obtained degree of quaternization was higher than what was expected (72%) [43], given that the procedure used entailed one reaction step plus two addition steps. The degree of substitution on hydroxyl groups was also quite different from what was predictable (48%–43%). As already pointed out by DiColo et al. [50], this result might be due to slightly different reaction conditions under which the methylation reaction was carried out.

After having identified the limits of the variables involved by preliminary experiments, a Doehlert experimental design was planned, as shown in Table 2, to investigate the effect of component amounts on nanoparticle characteristics. Taking into account the results of the previous investigation, described in Section 3.2., cisplatin–uronic molar ratio was fixed at 0.216, which was considered a suitable value to obtain a good drug loading without negative interference on nanoparticle formation. The experimental plan and the results of the experiments are shown in the same table.

The system under study was adequately described by a quadratic model, whose equations for D and Z are reported below (for Z the model was refined by eliminating a non-significant quadratic term in order to obtain an acceptable prediction power):

$$Y_1 = 261 + 5\ X_1 + 122\ X_2(^{**}) + 7\ X_1X_2 - 34\ X_1^2 + 34\ X_2^2$$

$$R^2 = 0.88,\ Q^2 = 0.91,\ RSD = 26\text{nm}$$

$$Y_2 = 54.2 - 9.1\ X_1(^{**}) + 8.4\ X_2(^{**}) + 11.9\ X_1X_2(^{*}) - 7.2\ X_1^2$$

$$R^2 = 0.84,\ Q^2 = 0.45,\ RSD = 3.5\text{mV}$$

where Y_1 is the response variable D, Y_2 the response variable Z, X_1 the alginate amount, X_2 the TMC amount (both to be introduced in the equations as coded values) and significant coefficients are marked by asterisks.

It can be seen from the corresponding contour plots depicted in Fig. 3 that nanoparticle size did not depend on the alginate amount in the sample, whereas a significant size increase was caused by raising the TMC amount. In the case of Z, the experimental design revealed a significant interaction between the amounts of alginate and TMC, indicating that an increase of the amount of TMC was important to achieving a higher level of Z when the amount of alginate in the sample was at a higher level. In other words, the Z value dropped if the TMC amount

Table 2
The experimental plan and results of a Doehlert design for two variables with four replicates at centre point

Alginate amount (X_1)		TMC amount (X_2)				
Real value (mg)	Coded value	Real value (mg)	Coded value	D (nm)	Z (mV)	PDI
1.05	1.000	3.50	0.000	230±3	40±3	0.33±0.06
0.94	0.500	5.67	0.866	390±3	58±1	0.18±0.07
0.60	−1.000	3.50	0.000	224±2	54±2	0.37±0.06
0.71	−0.500	1.34	−0.866	171±1	57±1	0.24±0.04
0.94	0.500	1.34	−0.866	173±3	33±1	0.05±0.08
0.71	−0.500	5.67	0.866	376±4	61±1	0.47±0.07
0.83	0.000	3.50	0.000	299±1	58±1	0.95±0.05
0.83	0.000	3.50	0.000	230±3	55±1	0.27±0.06
0.83	0.000	3.50	0.000	247±2	53±1	0.20±0.05
0.83	0.000	3.50	0.000	267±3	50±2	0.32±0.03

Coded values for the variables X_1 (alginate amount) and X_2 (TMC amount) are reported close to their real values. Composition is expressed by component amount (mg) in the final sample volume of 7.0 mL. Cisplatin–uronic acid molar ratio was fixed at 0.216.
Experimental data are represented as mean values±SD.

was at the lower level when the alginate amount was increased to the higher level.

Based on the results of the experimental design, two samples were prepared starting from different amounts of TMC with respect to the same amount of alginate. Having fixed the alginate amount at 0.9 mg, a sample (P1S) was obtained in the presence of a great excess of TMC, whereas a second sample (P2S) was prepared using an amount of TMC reduced by more than half. This was done in order to verify whether the drug release or the pharmacological activity of the particulate system might vary as a function of the weight ratio of TMC to alginate in the sample. Table 3 reports experimental data relating to the two samples, together with those relating to NPS sample discussed above. For all the samples, D and Z values were in line with those predicted by the mathematical model used to describe the system under study. Significantly higher mean values of D and Z were found for P1S with respect to P2S nanoparticles, thereby confirming that the use of a greater excess of TMC in preparing the sample induced the formation of particles bearing a greater number of TMC molecules entangled onto their surface. Mean Z values, as well as mean D values, were not significantly changed for nanoparticles redispersed in water; by contrast, SD was more than doubled for D values, thus indicating that redispersion caused a wider size distribution as a consequence of particle aggregation.

3.4. Nanoparticle yield, drug loading and yield of transformation

Nanoparticle yield, yield of transformation and drug loading are represented in Fig. 4.

Multiple comparisons, made by one-way ANOVA using post-hoc Bonferroni test, showed that nanoparticle yield was significantly higher for the negative particles (about 37% w/w) than for the positive ones; a significant difference between the two positively charged particles was also found (12% w/w for P1S, 16% w/w for P2S). For these nanoparticles, obtained using a TMC excess, the yield was similar to that found by Sandri et al., who obtained nanoparticles by ionotropic gelation between a trimethyl chitosan at 90% quaternization degree and tripolyphosphate [30]. It must be pointed out that the nanoparticle yield was calculated by referring to the total amount of polymers and drug used in the preparation. This led to an apparently unfavourable value, due to the excess of TMC used in formulations prepared to obtain the P1S and P2S samples. If the yield was calculated as the percent ratio between the particle weight and the starting cisplatin–alginate complex amount, it was equal to (60.4±7.3)% w/w for P1S particles and to (41.0±3.2)% w/w for P2S particles, whereas it raised to (43.3±0.5)% w/w for NPS particles (all data are expressed as mean±SD). In absolute terms, in the case of P1S particles, for example, about 2.5 mg of particles can be obtained from about 4 mg of complex.

The yield of transformation of the complex into nanoparticles was good and no significant differences were found between the positive nanoparticles and the negative ones. Calculations performed considering the percent ratio between the complex weight and the particle weight yielded the fraction of the complex incorporated into nanoparticles, which was

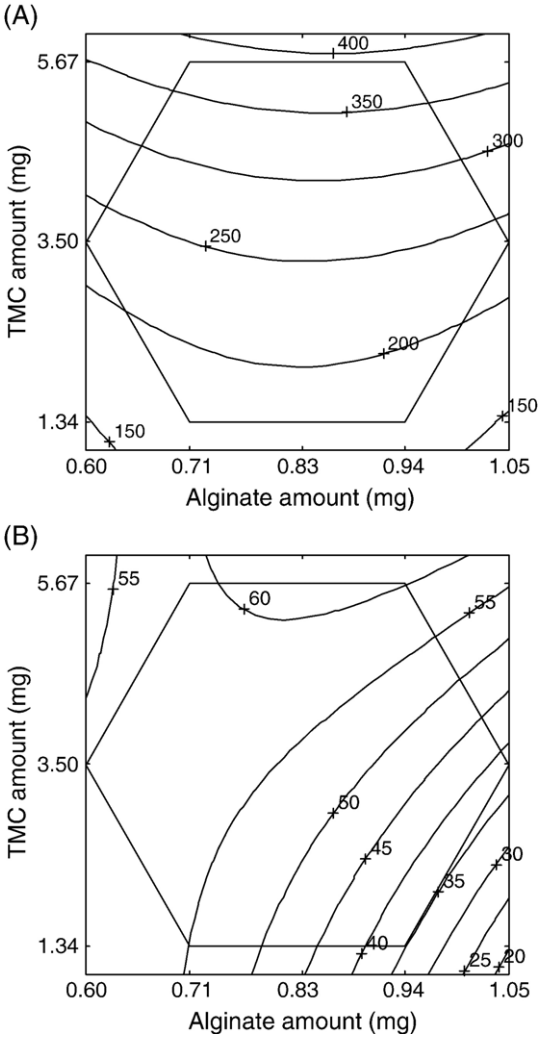

Fig. 3. Contour plots for D (nm) (A) and Z (mV) (B), derived from the Doehlert design represented in Table 2.

Table 3
Characteristics of TMC–alginate and chitosan–alginate nanoparticles

Sample	Cationic polymer type	Formulation		
		Cationic polymer amount (mg)	Alginate amount (mg)	Cationic polymer-alginate ratio (w/w)
P1S	TMC	5.0	0.90	5.6
P2S	TMC	2.0	0.90	2.2
NPS	Chitosan	2.5	12.0	0.21

Sample	*D* (nm)	*Z* (mV)	PDI
P1S	320±53	61±6	0.25±0.11
	280±120 [a]	60±6 [a]	0.43±0.04 [a]
P2S	188±16	43±7	0.18±0.15
	193±36 [a]	32±5 [a]	0.44±0.20 [a]
NPS	348±17	−34±5	0.27±0.15
	454±62 [a]	−40±4 [a]	0.38±0.28 [a]

Data are presented as mean±SD from genuine replicated samples (n=3). Formulation composition is expressed by component amount (mg) in the final sample volume (7.0 mL for TMC–alginate nanoparticles and 28.0 mL for chitosan–alginate nanoparticles). Cisplatin–uronic acid molar ratio in the complex was 0.216, corresponding to a cisplatin–alginate weight ratio equal to 0.33.
[a] After redispersion in water.

(expressed as mean±SD) (33±4)% for P1S particles, (84±2)% for P2S particles and (74±1)% for NPS particles. These data are particularly telling of the composition of nanoparticles made of alginate and TMC, because they show that P1S particles contained more TMC than P2S particles.

Drug loading ranged from 13% w/w to 21% w/w and was significantly lower for P1S nanoparticles. The values of drug loading registered for P2S and NPS nanoparticles were about one-half that of a micellar system based on aspartic acid [42,51], which was particularly high (about 39% w/w), and several times higher than that of PLGA-mPEG nanoparticles (1% w/w) [14]. In addition to a good drug loading, the particles described here present distinctive features when compared to already proposed cisplatin carrier systems. In fact, the nanoparticles are composed of alginate and chitosan or TMC and have a surface charge which is highly positive in particles made of alginate and TMC and markedly negative in particles made of alginate and chitosan. Thus, they substantially differ from the micellar system described in [42] as well as from the PLGA-mPEG nanoparticles described in [14], in terms of composition and charge, since such systems are characterized by a low negative

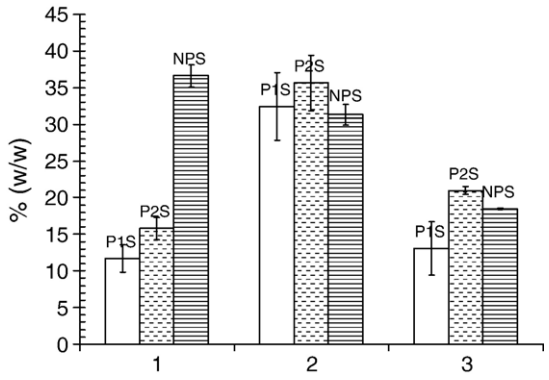

Fig. 4. Data on nanoparticle yield, yield of transformation of the complex into nanoparticles and drug loading. Experimental data are expressed as mean±SD from genuine replicated samples (n=3). 1—Nanoparticle yield (% w/w); 2—Yield of transformation (% w/w); 3—Drug loading (% w/w). P1S sample (TMC to alginate weight ratio=5.6), P2S sample (TMC to alginate weight ratio=2.2), NPS sample (Chitosan to alginate weight ratio=0.21).

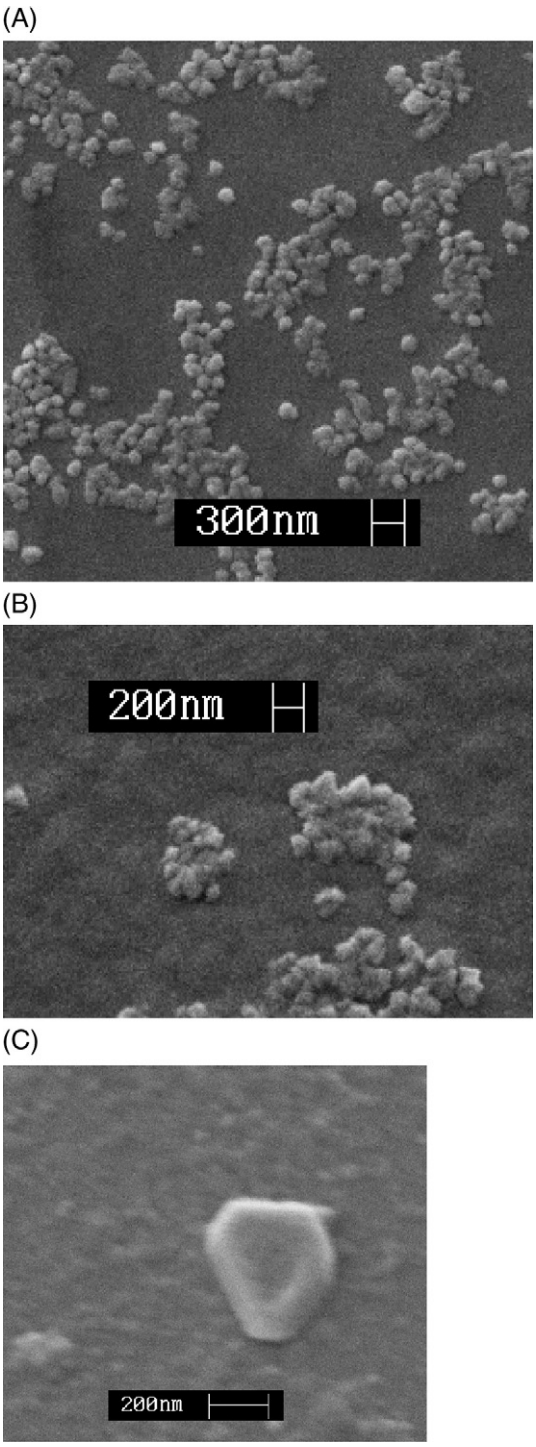

Fig. 5. SEM images of particles from P1S sample (A), P2S sample (B) and NPS sample (C). Photon correlation spectroscopy gave an average apparent diameter of (253±3) nm, (177±2) nm and (333±14) nm, respectively.

surface charge. With respect to the macromolecular cisplatin carriers, such as alginate or glutamate, the nanoparticles described here have, even when negatively charged, a higher charge density, due to their smaller size.

Particle images obtained by SEM from P1S, P2S and NPS samples, as shown in Fig. 5, indicated that experimental data from PCS were essentially confirmed in terms of sizes and size distribution. Particle agglomeration in the case of P2S sample might be due to the method of sample preparation for SEM, which involved a thermal treatment for solvent evaporation and might influence to a greater extent a sample of such a composition.

3.5. Cisplatin release from cisplatin–alginate complex and nanoparticles

The diffusion profile of free drug from a cisplatin solution, and release profiles from the cisplatin–alginate complex and from the different systems under study, is depicted in Fig. 6: 85% of total drug diffused from a cisplatin solution in PBS within 2 h, reaching 92% at 48 h, thereby indicating that cisplatin was retained by the dialysis membrane to a low extent.

As can be seen, nanoparticulate systems allowed controlling drug release significantly better than the cisplatin–alginate complex at a cisplatin–uronic acid molar ratio equal to 0.216. This latter gave a release curve indicating that the complex alone permitted a sustained release of cisplatin. The time at which 50% of total drug was released, corresponding to 5 h, was similar to that reported by Imai et al. [10] (7 h) for a complex at a cisplatin–uronic acid molar ratio equal to 0.1, without, however, any demonstration of the corresponding release curve.

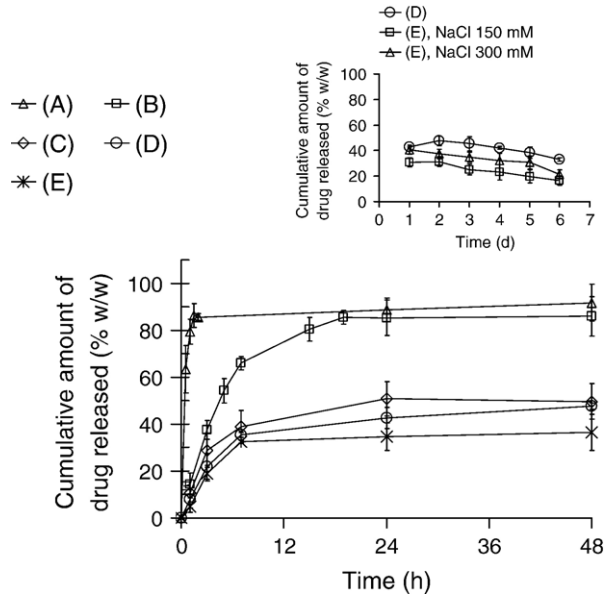

Fig. 6. Release profiles (PBS, pH 7.4) of cisplatin from a free drug solution (A), from cisplatin–alginate complex at 0.216 cisplatin–uronic acid molar ratio (B), and from nanoparticulate systems: P1S sample (TMC to alginate weight ratio=5.6) (C), P2S sample (TMC to alginate weight ratio=2.2) (D) and NPS sample (Chitosan to alginate weight ratio=0.21) (E). Long-term release profiles from NPS and P2S nanoparticles are depicted in the same figure. Data points represent the mean±SD from genuine replicated samples (n=2).

Some differences were found among the nanoparticle samples, which nonetheless released cisplatin quite similarly, regardless of their surface charge. While, after 24 h, the complex released 85% of the conjugated drug, only about 40% of total cisplatin was released by NPS sample and about 50% from P1S and P2S samples. This result confirms what was found by De and Robinson [15] when studying the drug release from nanoparticles made of chitosan and alginate at a weight ratio equal to half of the NPS sample presented here. With their findings, these authors asserted that the sodium ion concentration in the acceptor medium, constituted in their case by a solution at increasing sodium chloride concentration, controlled release capability, since the binding between the polyelectrolytes was competitively inhibited by sodium ions. A similar phenomenon, due to the presence of sodium and chloride ions in sufficient concentration, could be surmised to be active for positive particles, as well. In the case of the nanoparticles studied here, which bore cisplatin complexed with alginate, this preliminary event was needed to enable chloride ions to exert their action in replacing the good leaving groups carboxylate, thereby releasing the free drug from alginate macromolecules. The cumulative amounts of released drug decreased with time, after 48 h, as can be seen from the same Fig. 6, in which the long-term release curves for P2S and NPS particles are also shown. Due to the slightly acidic pH (approximately 5.5) of the solution during the formation of NPS nanoparticles, a competitive reaction for Pt between amino groups of chitosan and carboxylate ligands in the cisplatin–alginate complex was improbable, since amino groups were sufficiently protonated (over 90%) [52], thereby also allowing a stronger interaction with alginate. It cannot be ruled out that, during the release process, some cisplatin derived from particles owing to the effect of the salt ions might be sequestered by chitosan, since its amino groups are almost non-protonated at physiological pH. However, this event does not seem to be indicated by the long-term release study, where the release curve of P2S nanoparticles showed a drift towards lower values similar to that of the curve revealed by NPS nanoparticles. In fact, the same phenomenon was not possible in the case of TMC, which bore only trimethylated amino groups.

The curve obtained for the NPS sample, after increasing the sodium chloride concentration up to 300 mM, was shifted toward higher values, but not significantly (Fig. 6), indicating that cisplatin release was scarcely affected by a moderate increase of the amount of this salt in the medium. This finding is similar to that described for a chitosan–DNA complex, in relation to the effect of ionic strength on the ability of the salt to shield the chitosan–DNA interactions [52]. In fact, the cited study shows that a concentration of sodium chloride much higher than that predictable by reference [15] was needed to inhibit the electrostatic interaction between chitosan and DNA.

3.6. Antiproliferative activity, apoptosis and necrosis

The mean IC_{50}s calculated from the concentration–response curves obtained by the treatment of cell lines with cisplatin, nanoparticles and cisplatin–alginate complex are reported in

Table 4. Multiple comparisons by one-way ANOVA, using post-hoc Bonferroni test, showed that the complex and the positive P1S nanoparticles possessed an inhibition capability of cell proliferation similar to that of cisplatin in P388 and A2780 cells, while the P2S nanoparticles showed a significantly lower activity and the negative NPS nanoparticles were still less active. In the human A549 lung carcinoma cell line, the P2S nanoparticles displayed a better inhibiting activity than that of cisplatin, while the complex and the P1S nanoparticles maintained an activity similar to that of the reference drug; again, the negative NPS nanoparticles were less active. These results highlight the importance of both particle size and surface charge. In fact, NPS nanoparticles, which are characterized by the largest size and a negative zeta potential, were the least active on all cell lines. In the case of the P1S and P2S nanoparticles, the two factors seemed to play a compensatory role, also depending on the sensitivity of the cell line. In fact, the P2S and P1S nanoparticles respectively present smaller size associated with a lower positive zeta potential and higher size together with a higher positive zeta potential (Table 3). Since P1S particles always had an inhibitory activity similar to that of cisplatin, the higher positive zeta potential seems to play a major role, compensating for the higher particle size. On the other hand, the higher activity shown by P2S particles on the A549 cell line indicates that the smaller particle size can compensate the reduced effect of the lower zeta potential.

It is noteworthy that, when the nanoparticles were deprived of cisplatin, they mainly lost their antiproliferative activity. IC_{50}s of blank nanoparticles, expressed as equivalent cisplatin concentration, are also reported in Table 4. As can be seen from the data, blank NPS nanoparticles showed practically no activity in all cell lines under study. A similar behaviour was demonstrated by blank P2S particles, for which a definite IC_{50} value was found only in the case of P388 cell line, and was, however, ten times higher than that of drug loaded particles. Blank P1S particles were more active in inhibiting cell proliferation, when compared with the other nanoparticles, in particular in A549 cell line, where IC_{50} was only doubled with respect to drug loaded particles. In the other cell lines, however, blank P1S particles showed IC_{50} values 8–10 times higher than those of drug loaded particles. These results highlight the influence of the higher, potentially toxic, content of the TMC in the particles, as is the case of P1S nanoparticles compared to P2S nanoparticles, since these latter contain much less chitosan derivative.

Apoptosis was induced by both the complex and nanoparticles with a clear time dependence. After 3 days culture, P2S and P1S nanoparticles showed the highest relative amounts of apoptotic cells, although such amounts were not significantly different from that measured for cisplatin (Table 4). These results were consistent with what was found by the study of the inhibition of cell proliferation.

No differences existed in terms of necrotic PI^+ cells.

4. Conclusions

Nanoparticles with a positive or negative surface charge can be obtained through the electrostatic interaction of sodium alginate, previously complexed with cisplatin, with a cationic polyelectrolyte such as chitosan or *N*-trimethyl chitosan. This latter was employed in this study to overcome the difficulty entailed in the use of chitosan in excess due to its low solubility, and to guarantee a permanent positive surface charge on the obtained particles, even in a physiological environment. The use of statistical experimental design and response surface

Table 4

(A)

	IC_{50} (μM)			
Cell line	Cisplatin	Complex	NPS	Blank NPS
A549	6.87±1.51	5.25±0.90	13.9±2.9	>>59
A2780	1.56±0.32	1.30±0.31	3.87±0.33	>>59
P388	0.16±0.01	0.13±0.04	0.68±0.13	>>59

	IC_{50} (μM)			
	P1S	Blank P1S	P2S	Blank P2S
A549	5.06±0.44	11.5±4.4	4.01±0.69	>>27
A2780	1.70±0.30	13.3±4.5	2.22±0.67	>>27
P388	0.22±0.06	2.9±1.0	0.38±0.08	5.27±0.60

(B)

	Apo^+					
Day of culture	Control	Cisplatin	Complex	NPS	P1S	P2S
1	7.3±1.0	13.9±3.6	13.3±6.2	9.3±1.8	11.2±2.8	9.2±1.6
3	11.2±3.7	40.6±6.0	23.0±2.6	25.7±3.6	50.0±15.3	61.1±15.4

(A) Inhibition of cell proliferation by nanoparticles and cisplatin–alginate complex. IC_{50}s (μM) were obtained by the MTT assay. Each number represents the mean± SD of 8–9 IC_{50}s from independent experiments. (B) Apoptosis induced in A2780 cells by cisplatin–alginate complex and nanoparticles. Numbers express the mean± SD (n=3) of the relative amount (%) of apoptotic cells (Apo^+), expressed as the sum of early ($Ann\text{-}V^+/PI^-$) and late ($Ann\text{-}V^+/PI^+$) apoptotic cells, as determined by the cytofluorimetric analysis of Ann-V and PI. IC_{50} for blank nanoparticles is expressed as equivalent cisplatin concentration. P1S sample (TMC to alginate weight ratio=5.6), P2S sample (TMC to alginate weight ratio=2.2) and NPS sample (chitosan to alginate weight ratio=0.21).

methodology allowed for the study of the influence of component amounts and different cisplatin–uronic acid molar ratios on sizes and zeta potential of nanoparticles and for the identification of suitable formulations for the subsequent investigation. The studied systems, characterized by particle diameter ranging from 180 nm to 350 nm, showed a more sustained drug release profile in saline-buffered solution at pH 7.4 than the cisplatin–alginate complex. Preliminary pharmacological experiments on cytotoxicity *in vitro* indicated that the nanoparticles had a similar or slightly less activity compared to cisplatin on P388 and A2780 cell lines. On A549 human lung adenocarcinoma cell line, the nanoparticles with the smallest size and the lowest positive zeta potential, obtained from a smaller excess of TMC, displayed an enhanced cytotoxic activity compared to cisplatin. Moreover, nanoparticles behaved as the reference drug in inducing apoptosis in A2780 human adenocarcinoma cell line.

Overall, these results infer that the transformation of the cisplatin–alginate complex into nanoparticles is a quite promising approach. Particulate systems with characteristics that possibly enhance the therapeutic index of cisplatin could presumably be achieved even starting from other labile complexes, e.g., cisplatin–hyaluronic acid, but also from stabile complexes with other polymers of natural or synthetic origin, such as cisplatin–polyglutamic acid [8,37,38]. Work in this direction is in progress, as are studies in mice that endeavour to evaluate the *in vivo* antitumor activity of the particulate systems described here.

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