Polymer-based delivery vehicle for cisplatin

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ABSTRACT

Cisplatin is one of the most widely used agents in the treatment of a variety of tumors, but dose-limiting toxicities or intrinsic and acquired resistance limit its application. We report an unique strategy to deliver cisplatin to melanoma cells by sandwiching the drug between two oppositely charged polyelectrolytes: chitosan (CH) and carboxymethylcellulose (CMC), yielding cationic nanoparticles that present optimal colloid stability. Moreover, the relevance of CH structure was investigated by testing two CH with different degrees of acetylation of the glucosamine monomer. Both CH formed colloidal particles with similar sizes when mixed with CMC. However, only the CH with higher charge densities formed stable complexes with CMC to efficiently deliver the drug into cells. The cytostatic effects of free and encapsulated drug towards melanoma cells, were estimated by using the XTT assay. The results show that the chemotherapeutic drug is very cytotoxic in melanoma cells. The effectiveness of encapsulated drug against melanoma cells is approximately one order of magnitude greater than that of free drug. The strategy of delivering CisPt to melanoma cancer cells opens up avenues for systemic targeted therapy against this type of cancer using platinum drugs. More broadly, by targeting tumor-specific antigens, using similarly engineered nanoparticles, it may be possible to selectively deliver a therapeutic dose of platinum drugs to a myriad of cancers. Further studies with relevant animal models are needed.

Keywords: cancer, cisplatin, chitosan, polymeric nanoparticles.

1 INTRODUCTION

Among the methods for cancer treatment, chemotherapy using platinum drugs as antineoplastic agents has been an important technique for controlling many types of cancer including testicular, head, neck, and ovarian over the past few decades [1-3]. Cisplatin [*cis*-diamminedichloroplatinum(II)], the parent of all platinum drugs, is a highly effective antineoplastic agent that has been widely used for cancer therapeutics [4-5]. It preferentially binds to the N7 atoms of guanine bases in DNA double-helix strands, thereby preventing the strands from uncoiling and separating. This prohibits the division of the cells and may ultimately results in cellular apoptosis [6-8].

Although cisplatin is very potent and a sucessful antineoplastic agent, several side-effects limit its use in cancer treatment, including nephrotoxicity, cumulative neurotoxicity, ototoxicity (hearing loss), and severe emetogenesis (vomiting) [9]. In addition, the rapid inactivation due to tissue and plasma proteins and the occurrence of inherent or treatment-induced resistat tumor cells limit the therapeutic efficacy of cisplatin [10]. One of the strategies to reduce the systemic toxicity and to prevent inactivation of platinum drugs in the bloodstream and tissue fluid is the developtment of drug delivery systems [11], such as polymer-based nanoparticles, lipid-based nanoparticles (liposomes, nanoemulsions, solid lipid nanoparticles (SLN), biomimetic nanoparticles), self-assembly nanostructures such as micelles and dendrimers-based nanostructures among others [12]. However, loading cisplatin into polymeric nanoparticles is challeging because of its poor solubility in organic solvents and only partial solubility in water [13]. Despite of that, the use of nanoparticles to sensitize tumor cells to cisplatin *in vitro* and *in vivo* has been recently described [14-16]. In these studies, cisplatin-encapsulated nanoparticles were used to control the release of cisplatin into the cisplatin-resitant human prostate cancer cells [15]; observed the effects were cell-line specific [16].

In the work described here, cisplatin was sandwiched between two oppositely charged polyelectrolytes - chitosan (CH) and carboxymethylcellulose (CMC), and we hypothesized that the carrier is able not only to load cisplatin into polymeric nanoparticles but also to control drug release kinetics from the nanocarriers. This would reduce the amount of drug leaching out of the nanoparticles during their circulation in the blood (pH = 7.4) and enable intracellular drug release after endocytosis of the nanoparticles by the melanoma cells. To this end, two CH of different, well-defined, chemical structures were purchased in order to establish structure-property relationships with regard to complex formation and *in vitro* drug delivery efficiency. We demonstrated that only the drug-CH-CMC carrier with the high charge density CH had

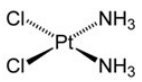
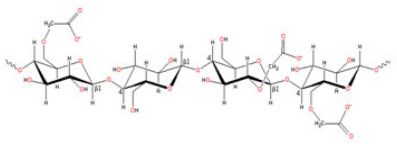
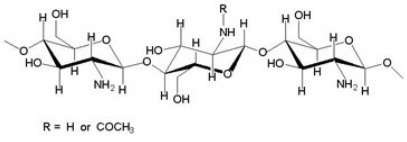
well controlled drug loading yield and potent cytotoxicity against melanoma cancer.

2 MATERIALS AND METHODS

2.1 Materials

Chemical structures of *cis*-diamminedichloroplatinum (II) (cisplatin, Cis), chitosan (CH) and carboxymethylcellulose (CMC) (Table 1) Chitosans having well-defined degrees (CH25 and CH75) of acetylation were obtained from Sigma-Aldrich. Chitosan was purified before use by dissolving in acetic acid 1%, followed by precipitation with NaOH (final pH= 8.0). Subsequently, the pellet was thoroughly washed with distilled water and rinsed twice with ethanol for about 4h. The product was milled and the chitosan powder was dried at 60 ºC, overnight. Finally, the product was stored at 4ºC. CMC and Cis were purchased from Fluka-Biochimika and Sigma-Aldrich, respectively, with the highest purity available.

Table 1: Chemical structure of compounds used to formulate cisplatin.

Name and abbreviation	Chemical structure
cis-diamminedichloroplatinum (II) (cisplatin, Cis)	
carboxymethylcellulose (CMC)	
chitosan (CH)	

2.2 Preparation and characterization of cisplatin-loaded nanoparticles

CHs stock solutions (1 gL^{-1}) were prepared by dissolving chitosan in Milli-Q water at pH= 3.0. CMC stock solution (0.4 gL^{-1}) was prepared by dissolving the salt in Milli-Q water at pH 6.3. Cis stock solutions (1, 5 or 10 mM) were dissolved and incubated in the dark overnight at 37 ºC and diluted in the dispersion to yield the desired final concentration. Polyelectrolytes and drug stock solutions were sterilized by sterile filtration. The stock solution of Cis was added to CMC solution. And the CH solution was added. Final concentrations of both polyions was 0.1 gL^{-1}. Nanoparticles sizes and zeta-potentials were determined by means of a ZetaPlus Zeta-Potential Analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) equipped with a 570 nm laser and dynamic light scattering at 90° for particle sizing (Z). The zeta-average diameters referred to in this work from now on should be understood as the mean hydrodynamic diameters D_z. Zeta-potentials (ζ) were determined from the electrophoretic mobility μ and Smoluchowski's equation, $\zeta = \mu\eta/\varepsilon$, where η and ε are medium viscosity and dielectric constant, respectively. All D_z and ζ were obtained at 25°C, 20 minutes after mixing. Also, nanoparticles size was measuring using atomic force microscopy (AFM). AFM topographic images were obtained using a PicoSPM-LE molecular imaging system with cantilevers operating in the intermittent-contact mode (AAC mode), slightly below their resonance frequency of approximately 290 kHz in the air. All topographic images represent unfiltered original data and refer to scan areas of (5 × 5) μm^2 and (1 × 1) μm^2 with a resolution of (512 × 512) pixels. At least two samples of the same material were analyzed at different areas of the surface. Image processing and the determination of the root-mean-square (rms) roughness were performed using the PicoScan 5.3.2. software. Optical microscopy was performed by means of a NAVITAR zoom lens system and Ultra TV software.

2.3 Drug loading studies

To measure the drug loading yield, 5 mL of the prepared Cis-CMC-CH25 or Cis-CMC-CH75 dispersions were loaded into several semi permeable membranes. The nanoparticles were then dialyzed against pH=3.6 and 6.3 Milli-Q water. After 1 hour, 100 μL of nanoparticles dispersions from three membranes dialysis were collect separately for drug quantification and evaporated at 100 ºC. An equal volume of dimethylformamide was added to the tube to maintain the volume. The concentration of cisplatin was quantified using a UV-spectrophotometer at 310 nm.

2.4 Cell viability assays

The human melanoma cell line SK-mel-28 was maintained in T-75 flasks at 37 ºC in a 95% humidified atmosphere and 5% CO_2 in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Cytotoxicity of Cis-CMC-CH25 or Cis-CMC-CH75 nanoparticles was assessed against SK-mel-28 human melanoma cell line using the XTT assay. First, SK-mel-28 melanoma cells were seeded (2 x 10^4 cells per well) in 12-well plates and incubated for 24 hours. Next, the medium was replaced with 250 μL of fresh medium and incubated wit 250 μL of Cis-CMC-CH25 or Cis-CMC-CH75 nanoparticles for 3 hours. Then, 500 μL of DMEM supplemented with 20% fetal calf serum was added per well. The plates were incubated for 24 hours and measured with XTT reagent following a protocol provided by the

manufacturer. Fresh cell media and CMC-CH25 or CMC-CH75 was used as negative control. Free cisplatin at various concentrations was used as positive control.

3 RESULTS AND DISCUSSION

The chitosan structure can be modified by changing the degree of acetylation of glucosamine monomers, ie the charge of the polymer. The chitosans (CH) were defined by numbers. The number gives the degree of de-acetylation in %. Two chitosans with degrees of de-acetylation of 25 or 75% of the glucosamine monomers having a primary amine were produced to investigate relevant structure property relationships. The CHs were complexed with carboxymethylcellulose (CMC) at charge ratio 1:1 and tested for complex stability and for delivery of the chemotherapeutic agent cisplatin (Cis) efficiency in melanoma cells.

Nanoparticles were designed and prepared by the complex coacervation method [17, 18] as shown in Figure 1A. Nanoparticles are formed by electrostatic interactions between the polycation CH and the polyanion CMC. Briefly, when the Cis-containing CMC and CH solutions are mixed, Cis within a CMC-CH25 or CMC-CH75 nanoparticles spontaneously form, generating particles with a positive surface charge in both cases. The properties of the nanoparticles were characterized by dynamic light scattering to give the size distribution, mean diameter and polydispersity of each preparation. Also, the surface morphology and size of CMC-CH25 nanoparticles was evaluated by atomic force microscopy (AFM). Both chitosans formed colloidal particles of similar sizes when mixed with CMC (Figure 1B and 1C). In agreement with the size measured by dynamic light scattering, CMC-CH25 particles presented a diameter of approximately 200 nm measured by AFM (Figure 1E, 1F and 1G). To optimize the size and loading, a series of encapsulated nanoparticles were prepared, varying the concentration of Cis (Figure 1D).

The loading efficiencies of Cis at different concentrations are shown in Figure 1D and both nanoparticles incorporated the same amount of drug. The polydispersity of Cis-CMC-CH25 particles slightly decreases with the concentration of Cis (data not shown). However, the size of the particles decreased from 196 to 149 nm when 1 mM of Cis was added and increased from 196 to 227 or 264 nm at 5 or 10 mM of Cis, respectively. As nanoparticles with size smaller than 150 nm are more effective at evading uptake by macrophages and remain longer in the bloodstream [19, 20, 21], for all studies we used encapsulated nanoparticles having approximately 80% of Cis-incorporated and a size of approximately 142 nm, after dialysis process to remove the free drug.

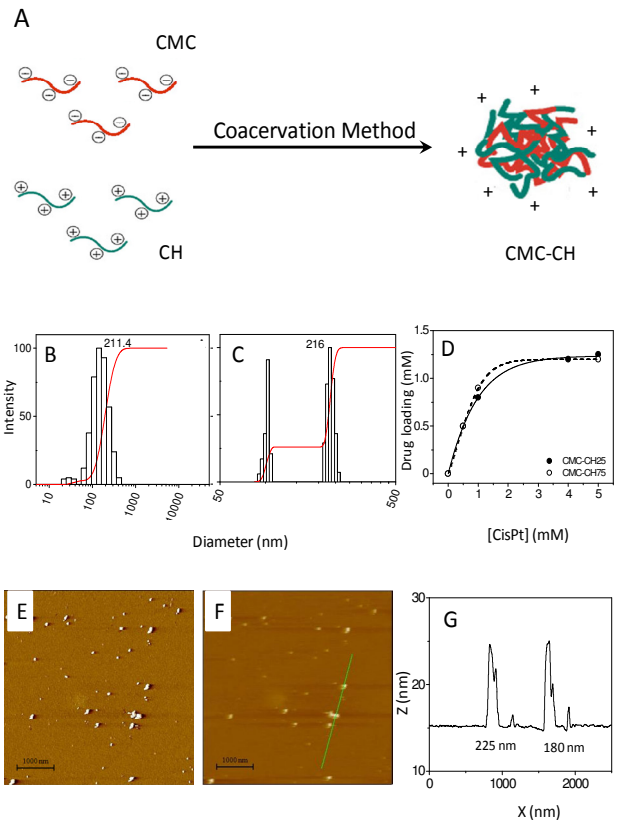


Figure 1: Nanoparticles production. (*A*) When aqueous solutions of a polycation and polyanion are mixed, polyelectrolytes complexes form spontaneously. The interaction is entropically driven. For this drug delivery system, an excess of polycation is used, generating particles with a positive surface charge. *(B and C)* Size of CMC-CH25 or CMC-CH75 nanoparticles, respectively. (*D)* Loading of Cis in the CMC-CH25 (closed circles) or CMC-CH7 (open circles) nanoparticles. (*E, F and G)* Atomic force micrograph of polyelectrolytes complexes, in this case CMC-CH25 nanoparticles. Scale bar = 1.000 nm.

Lastly, we examined the *in vitro* cytotoxicity assays to evaluate the anti-cancer potential of Cis-encapsulated nanoparticles towards melanoma cells and directly compare its efficacy to that of Cis. As shown in Figure 2B, Cis-CMC-CH75 is highly cytotoxic to the melanoma cells, having an IC_{50} value of 0.08 µM. Under the same conditions, the Cis-CMC-CH25 nanoparticles (Figure 2A) have an IC_{50} value of 0.5 µM, and for cisplatin the value with this cell is 0.8 µM. Free cisplatin has an IC_{50} of 0.8 µM with melanoma cells. These results demonstrate that Cis-CMC-CH75 delivers the drug more efficiently and is 10 times more toxic than free cisplatin in the SKmel28 cells, indicating this nanoparticle's potential to treat human cancer.

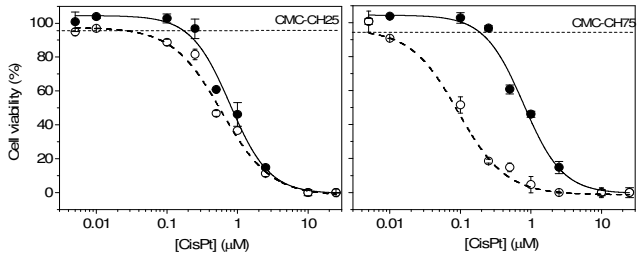


Figure 2: Cellular viability curves of melanoma cells treated with Cis-CMC-CH25 (A) or Cis-CMC-CH75 (B) nanoparticles (open circles) or free drug (closed circles). Cells (20000) were plated in 12-well plates with 1 mL medium per well. After 24 hours, various concentrations of Cis-CMC-CH nanoparticles or Cis were added, and then cells were incubated at 37 ºC for 24 hours. Before the cell viability measurement, XTT solution was added to each well and incubated for 1 hour. Cell viability was measured using spectrophotometer.

We found that stable nanoparticles capable of delivery cisplatin to melanoma cells *in vitro* were obtained only when the CH had a high density of positive charges. This agrees with the fact that complexes between cationic polymers and anionic pDNA are predominantly formed by ion-ion interaction [22]. The high physical stability of the CMC-CH75 has important implications. First, the chemotherapeutic agent was protected from rapid inactivation in the medium. Second, the release of cisplatin from the CMC-CH75 nanoparticles will probably occur at sites where CH-degrading enzymes are present. In animal cells, CH is degraded into oligomers by lysozyme, and then further degraded by N-acethyl-glucosaminidase [23]. Both of these enzymes are present in the endosomal/ lysosomal vehicles, which means that the degradation and release of Cis will start immediately after endocytosis of the cis-CMC-CH nanoparticles. Moreover, upon internalization, nanoparticles can cause a surge in intracellular drug concentration that possibly overhelmed some chemoresistance mechanisms of tumor cells [24].

4 CONCLUSIONS

In conclusion, the results of the present study defined the structural requirement on CMC-CH nanoparticles as a drug delivery system and indicate that drug-CMC-CH75 nanoparticles possess characteristics of an ideal nanocarrier. Cis-CMC-CH75 nanoparticles have well-controlled Cis loading yield, leading to enhanced *in vitro* cytotoxicity against tumor cells compared to free cisplatin. These nanoparticles can potentially minimize the drug loss during their circulation in he blood, where the pH is neutral, and trigger rapid intracellular drug release after endocytosis by cancer cells. This characteristic may suppress cancer cell chemoresistance and improve the therapeutic efficacy of the drug payload.

5 REFERENCES

[1] L. Kelland, Nat. Rev. Cancer 7, 573, 2007.
[2] T. Boulikas and M. Vougiouka, Oncol. Rep. 11, 559, 2004.
[3] B.J. Roth, Semin. Oncol. 23, 633, 1996.
[4] Z. H. Siddik, Oncogene 22, 7265, 2003.
[5] D. Wang and S.J. Lippard, Nat. Rev. Drug. Discov. 4, 307, 2005.
[6] M. Galanski, V.B. Arion, M.A. Jakupec and B.K. Keppler, Curr. Pharm. Des. 9, 2078, 2003.
[7] A. Pasini and F. Zunino, Angew. Chem., Int. Ed. Engl. 26, 615, 1987.
[8] E. Wong and C.M. Giandomenico, Chem. Rev. 99, 2451, 1999.
[9] V. Pinzani, F. Bressolle, I.J. Haug, M. Galtier, J.P. Blayac and P. Balmes, Cancer Chemother. Pharmacol. 35, 1, 1994.
[10] Y.O. Ho, S.C. Au-Yeung and K.K. To, Med. Res. Rev. 23, 633, 2003.
[11] K.J. Harrington, K.N. Syrigos and R.G.Vile, J. Pharm. Pharmacol. 54, 1573, 2002.
[12] D. Peer, J.M. Karp, S. Hong, O.C. Farokhzad, R. Margalit and R. Langer, Nat. Nanotech. 2, 751, 2007.
[13] V.P. Torchilin, Adv. Drug Deliv. Rev. 58, 1532, 2006.
[14] S. Basu, R. Harfouche, S. Soni, G.Chimote, R.A. Mashelkar, A. Raghunath A. and S. Sengupta. Proc. Natl. Acad. Sci USA 106, 7957, 2009.
[15] S. Dhar, F.X. Gu, R. Langer, O.C. Farokhzad and S.J. Lippard, Proc. Natl. Acad. Sci USA 105, 17356, 2008.
[16] I.H.L. Hamelers, R.W.H.M. Staffhorst, J. Voortman, B. de Kruijff, J. Reedijk, P.M.P. van Bergen en Henegouwen and A.I.P.M. de Kroon, Clin. Cancer Res. 15, 1259, 2009.
[17] L. Battaglia, M. Gallarate, R. Cavalli and M. Trotta, J. Microencapsul. 27, 78, 2010.
[18] Y.C. Toh, S.Y. Ho, Y. Zhou, D.W. Hutmacher and H. Yu. Biomaterials 26, 4149, 2005.
[19] F. Alexis, E. Pridgen, L.K. Molnar and O.C. Farokhzad, Mol. Pharmaceut. 5, 505, 2008.
[20] D.B.Buxton, Nanomedicine 4, 331, 2009.
[21] M.A. Dobrovolskaia, P. Aggarwal, J.B. Hall and S.E. McNeil, Mol. Pharmaceut. 5, 487, 2008.
[22] M.X. Tang and F.C. Szoka. Gene Therapy 4, 823, 1997.
[23] R.J.Nordtveit, K.M. Varum and O. Smidsrod. Carbohydr. Polym. 29, 163, 1996.
[24] M.M. Gottesman, Annu. Rev. Med. 53, 615, 2002.