Supramolecular assemblies of cisplatin and polyelectrolytes: preparation, characterization and activity against cancer cells

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ABSTRACT

Nanoformulation of cisplatin (Cis) is important to circumvent its high toxicity against normal cells. In this work, Cis cationic nanoparticles were prepared by the self-assembly of drug and oppositely charged polyelectrolytes (carboxymethylcellulose, CMC; chitosan, CH; polyethyleneimine, PEI) over a range of Cis concentrations ([Cis] = 25-250 nM; [CMC] = [CH] = 0.1 mg/mL). The nanoparticles thereby obtained exhibited 70-200 nm mean diameter and 32 -48 mV of zeta-potentials. The effect of increasing the positive charge on particles was increasing cell death by apoptosis. In addition to the experiments with cancer cells, other cells lines were tested, which presented different sensibility to the drug. Against XPA and MRC5-SV cells, which are deficient and proficient cell lines in nucleotide-excision repair pathway, respectively, the formulations exhibit differential cytotoxicity against the cells. Up to 250 nM Cis in the CMC/CH particles, the deficient cells were efficiently killed by apoptosis whereas the proficient cells were barely killed. The novel formulations have potential to deliver Cis *in vivo*.

Keywords: carboxymethylcellulose, chitosan, cisplatin, nanoparticles, activity against melanoma cells

1 INTRODUCTION

Cis-diamminedichloroplatinum(II) or cisplatin (Cis) is a highly effective antineoplastic drug that has been widely used in cancer chemotherapy [1]. Cis mechanism of action involves its binding to the N7 atoms of guanine bases in DNA double-helix strands, which prevents the uncoiling and the separation of the DNA strands taking place during DNA replication and cell division [2]. Ultimately, the cancer cell dies via apoptosis [2]. However, drug toxicity to normal cells and acquired resistance are limiting factors against successful therapy with Cis [2].

Several delivery strategies have been developed to improve the therapeutic index of drugs in general [3-5] and cisplatin in particular such as encapsulation of Cis nanoparticles by phospholipids [6-7] or polyelectrolytes and/or polymers [8-12]. Recently, the cross-linking between cisplatin and two oppositely charged polyelectrolytes, chitosan (CH) and carboxymethylcellulose (CMC) was reported suggesting a novel formulation for the drug based on nanoparticles [12]. In this work, a complete physico-chemical characterization of the CMC-Cis-CH nanoparticles is presented. The quantitative Cis incorporation as a function of added drug and the drug release profile as a function of time are reported. Furthermore, the loaded nanoparticles are shown to be colloidally stable from the narrow size distributions and the reduction of mean particle size and polydispersity. At low pH, for CH25, a chitosan with a low degree of deacetylation, the hydrogen bonding interaction might explain the crosslinking between CMC and CH25 as hypothesised in Fig. 1.

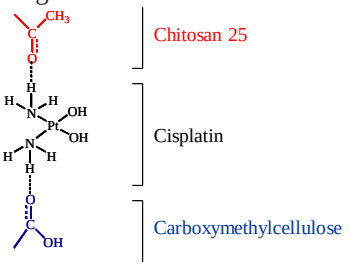

Figure 1: Hydrogen bonding determining the self-assembly of CMC, cisplatin and CH25.

The results for a chitosan with a lower degree of acetylation (CH75) at pH 6.3 further corroborated the cross linking hypothesis at low pH suggesting the predominance of the electrostatic attraction between CH75 and CMC for maintainance of the CMC-Cis-CH75 assembly. The Cis release determined for both chitosans revealed that only the electrostatic attraction between CMC and CH was able to produce suitable nanoparticles for a controlled release of Cis over time at pH values close to the physiological one. From the CMC-Cis-CH75 nanoparticles, the Cis release took place over the first 48 h whereas from the CMC-Cis-CH25 nanoparticles, the Cis release was practically instantaneous. At last, the treatment of XPA cells with the CMC-Cis-CH75 nanoparticles, increased cell death by apoptosis by 3 times as compared to the treatment of the same cells with the free drug.

2 MATERIALS AND METHODS

2.1 Materials

CH25 was obtained from Sigma and purified before use by adding acetic acid 1%, precipitating with NaOH (final pH 8), thorough washing of the precipitate with distilled water, rinsing twice with ethanol, milling and drying (60 ºC /12 h) for storage at 4ºC. CH75 was also obtained from Sigma, with the highest purity available, and used as such. CMC and Cis were purchased from Fluka-Biochimika and Sigma, respectively, with the highest purity available.

2.2 Preparation and characterization of cisplatin-loaded nanoparticles from dynamic light-scattering and atomic force microscopy

CMC stock solution (0.4 gL^{-1}) was prepared in Milli-Q water. CH stock solutions (1 gL^{-1}) were prepared in Milli-Q water previously adjusted to pH 3 with acetic acid. Cis stock solutions were prepared at 1, 5 or 10 mM Cis, kept in the dark and incubated overnight at 37 ºC before use. All stock solutions were filtered through a 200 µm cut-off membrane for sterilization. For preparing the nanoparticles, firstly, an aliquot of the Cis stock solution was added to the CMC solution. Thereafter, the CH solution was added. Final CMC and CH concentrations were 0.1 gL^{-1}. Nanoparticles size distribution, particle size (Dz), zeta-potential (ζ) and polydispersity (P) 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° [13]. The zeta-average diameter (Dz) referred to in this work is to be understood as the mean hydrodynamic diameter. The zeta-potential was 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, 1 h after preparing the nanoparticles. The nanoparticles were also visualized from atomic force microscopy (AFM) using a PicoSPM-LE molecular imaging system with cantilevers operating in the intermittent-contact mode, slightly below their resonance frequency of approximately 290 kHz in the air. Topographic and phase contrast 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. Image processing was performed by using the PicoScan 5.3.2. software.

2.3 Determination of drug loading

Several aliquots of the CMC-Cis-CH25 or CMC-Cis-CH75 dispersions with 5 mL each were placed inside cellulose acetate dialysis bags. Dialysis was performed at two pH values, 3.6 and 6.3, in order to establish the dependence of Cis entrapment on CH acetylation degree and pH. One should notice that a low positive charge on CH is impeditive to the proper formation of CMC-CH nanoparticles. The first dialysis took place for 1 h against 2 L of Milli-Q water previously adjusted to pH=3.6. Thereafter, the same dispersion was dialysed (1 h) against 2 L of Milli-Q water at pH 6.3. After each dialysis, aliquots of 100 µL fo the dispersions were withdrawn from the bag and used for spectrophotometric determination of Cis concentration as follows. Samples were evaporated at 100 ºC, allowed to achieve the room temperature and added of 100 µL of dimethylformamide (DMF) before reading absorbance at 306 nm. From appropriate standard curves of absorbance at 306 nm and [Cis] and the absorbance reading, Cis entrapment (%Inc) in the nanoparticles could be calculated as $\% \text{ Inc} = 100[\text{Cis}]_{\text{after dialysis}} / [\text{Cis}]_{\text{before dialysis}}$.

2.4 Determination of drug release

Seven aliquots of CMC-Cis-CH nanoparticles in water at pH 3.6 (1 mL) were dialysed against 2 L of phosphate-buffered saline (PBS) at 25 °C/ pH 7.4 and 10 µL samples were withdrawn from the bags at given instants over a range of time (0-72 h). These 10 µL were used to determine Cis concentration by adding 500 µL of 1.2 mg⁄mL OPD in DMF (3h /100 °C) for complexation between Cis and ortho-phenylenediamine (OPD) followed by absorbance reading at 710 nm [14]. Cis release (%Rel) from the nanoparticles was calculated from:

$\% \text{ Rel} = 100 ([\text{Cis}]_{\text{before dialysis}} - [\text{Cis}]_{\text{after dialysis}}) / [\text{Cis}]_{\text{before dialysis}}$

2.5 Cell viability assays

The human melanoma SK-mel-28, XPA and MRC5 cell lines were maintained in T-75 flasks (Corning Constar, Cambridge MA) 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 bovine serum. Cytotoxicity of CMC-Cis-CH25 or CMC-Cis-CH75 nanoparticles was assessed against the three quoted cell lines using the colorimetric assay (XTT based) for the quantification of cell proliferation and viability (Roche, Mannheim, Germany). Firstly, cells were seeded (2 x 10^4 cells per well) in 12-well plates and incubated for 24 hours. Thereafter, the medium was replaced with 250 µL of fresh medium and incubated with 250 µL of CMC- Cis-CH25 or CMC-Cis-CH75 nanoparticles for 3 hours. Then, 500 µL of DMEM supplemented with 20% fetal bovine serum was added per well. The plates were incubated for 24 hours before following the Roche protocol. Fresh cell media and unloaded CMC-CH25 or CMC-CH75 were also tested as the negative controls. Free cisplatin solutions over a range of concentrations were used as positive controls.

2.6 Determination of sub-G1 nuclei in XPA cells from flow cytometry

Determination of sub–G1 nuclei was performed by flow cytometry 24 h after treating XPA cells with cis-

nanoparticles or cis alone. Cells were collected and centrifuged at 1,000 rpm for 5 min. Pelleted cells were fixed with 70% ethanol and stored for up to 1 week at −20°C. Immediately before analysis, cells were treated with RNase (0.03 mg/mL) and subsequently stained with propidium iodide (16.5 mg/mL) in PBS. Samples were then transferred to 96-well plates, and propidium iodide fluorescence was measured by flow cytometry (Guava EasyCyte plus). For each sample, 10,000 cells were analyzed and the results were shown as percentage of subdiploid nuclei (Guava Express Pro Analysis), which represent apoptotic cells [15].

3 RESULTS AND DISCUSSION

The major interaction between the polycation CH and the polyanion CMC is the electrostatic attraction which drives the formation of nanoparticles such as those shown in Figure 2. Sizing for these unloaded nanoparticles was determined both by dynamic light-scattering and AFM, with good agreement between results obtained from both techniques (Figure 2 B, C, E, F and G). Figure 2 D shows the incorporation (%INC) of cisplatin in the nanoparticles as a function of the final cisplatin concentration. At pH 3.6, the hyperbolic profile of drug incorporation into the nanoparticles revealed that the nanoparticles become saturated with the drug from ca. 2 mM Cis with 1.5 mM Cis as the maximal incorporation. %INC is very similar for both chitosans at pH 3.6 (Figure 2 D).

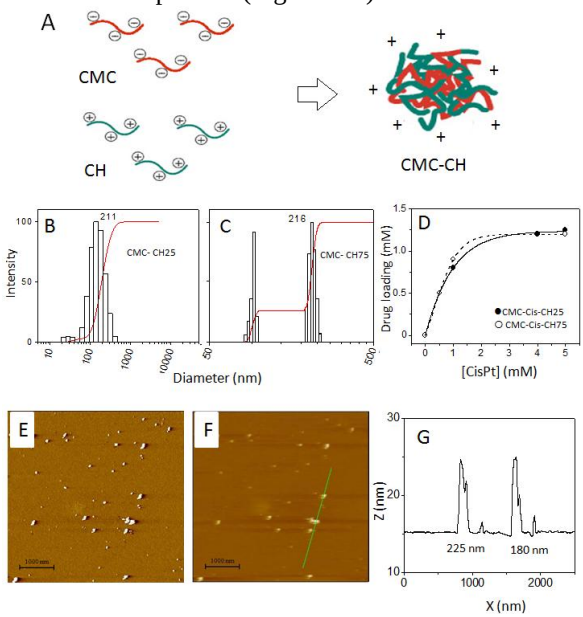

Figure 2: Preparation of CMC-CH nanoparticles (A), size distributions from dynamic light-scattering for CMC-CH25 (B) or CMC-CH75 (C). Phase contrast AFM (E) and topographic AFM images of CMC-CH25 nanoparticles (F) and the cross-section profile from the green line (G). Cis loading into CMC-Cis-CH nanoparticles at pH 3.6 as determined from dialysis. Final [CMC] and/or [CH] were 0.1 mg/mL.

The interaction between Cis and CMC is probably driven by: 1) hydrogen bonding between CMC carbonils and hydrogens from amino moieties in Cis; 2) electrostatic attraction between positively charged Cis and dissociated carboxyls in CMC. The addition of CH to the CMC-Cis assembly results in formation of nanoparticles also due to; 1) hydrogen bonding between chitosan acetils and the amino moieties in Cis; 2) the electrostatic attraction between CMC-Cis and CH.

The controlled release of Cis at pH 6.3 was strictly dependent on the use of a chitosan with a high degree of deacetylation (CH75). At pH 6.3, for CMC-Cis-CH75 nanoparticles, %INC remains equal to ca. 20 % whereas for CMC-Cis-CH25 it decreases to 5 % (Table 2). This suggests the importance of net CH positive charge in keeping the integrity of the nanoparticle. At pH 6.3, the comparatively low density of positive charges in CH25 results in a lower zeta-potential for the CMC-Cis-CH25 nanoparticle and in a lower %INC of the drug (Table 2). The CH25 deprotonation at pH 6.3 induces looser packing in the nanoparticle with loss of Cis to the bulk solution. Curiously, the drug systematically contributed to the occurrence of a highly homodisperse population of nanoparticles as depicted from the very low polydispersity obtained both at pH 3.6 and 6.3 (Table 2), possibly due to crosslinking that occurs between the oppositely charged polyelectrolytes when Cis is present. At 1 mM Cis and pH 3.6, the effect of Cis on CMC-Cis-CH25 nanoparticles is clearly seen as a narrowing of the size distributions (Figure 3).

Table 1. Effect of pH, cisplatin incorporation (% Inc) and CH degree of acetylation on physical properties of CMC-CH nanoparticles (Dz, zeta-potential and polydispersity).

Dispersion	pH	[Cis]/mM	Dz ±δ/nm	ζ±δ/mV	Polydispersity	% Inc
CMC-CH25	3.6		189±1	45±2	0.191 ± 0.007	-
CMC-cis-CH25		10	199±1	46±3	0.125 ± 0.008	21
CMC-CH75		-	151±1	43±2	0.184 ± 0.004	-
CMC-cis-CH75		10	206±2	45±3	0.057 ± 0.018	20
CMC-CH25	6.3	-	195±2	40±2	0.171 ± 0.003	-
CMC-cis-CH25		10	186±1	32±2	0.158 ± 0.008	5
CMC-CH75		-	164±1	41±2	0.105 ± 0.014	-
CMC-cis-CH75		10	207±2	42±2	0.065 ± 0.017	19

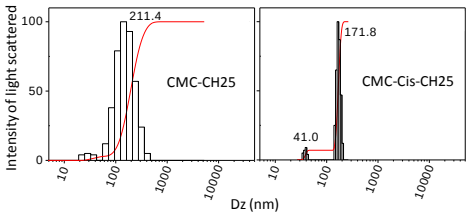

Figure 3: Size distributions for CMC-CH25 and CMC-Cis-CH25 nanoparticles at 0.1 gL^{-1} CMC and CH25, 1 mM Cis.

At pH 7.4, the Cis release (%REL) from the nanoparticles is also strictly dependent on the CH degree of acetylation. The loose polymeric network in the CMC-Cis-CH25 nanoparticle yields a rapid drug release whereas the tight packing in the CMC-Cis-CH75 assembly causes the controlled release of Cis (Figure 4).

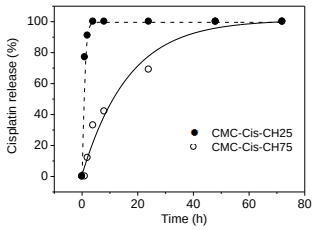

Figure 4: Cisplatin release *in vitro* from CMC-Cis-CH nanoparticles determined by means of dialysis in phosphate buffer saline at pH 7.4. In the nanoparticles, final concentrations before dialysis were 0.1 mg/mL CMC, 0.1 mg/mL CH and 1.25 mM Cis. Aliquots from the dispersion inside the dialysis bag were taken at regular times and had their Cis concentration determined from UV-Vis spectroscopy after Cis complexation with ortho-phylenediamine (OPD).

CMC-Cis-CH75 dispersion is highly cytotoxic to melanoma cells, with 0.08 μM Cis as the IC_{50} dose (Figure 5, middle). Free cisplatin against melanoma cells has an IC_{50} of 0.8 μM (filled circles in Figure 5). In contrast, the CMC-Cis-CH25 nanoparticles show practically the same cytotoxicity as the free drug (Figure 5, left). This agrees with the very loose packing of these nanoparticles at pH values close to the physiological one. Thereby, the drug is released even before these nanoparticles enter the cell. CMC-Cis-CH75 nanoparticles are 10 times more toxic than the free drug against the cancer cells and induce 3 times higher percentiles of sub-G1 nuclei in XPA cells (Figure 5, right). Thus, the mechanism for cell death at least in part, involves apoptosis.

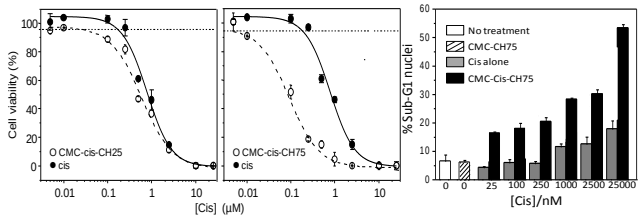

Figure 5- The effect of CMC-Cis-CH25 or CMC-Cis-CH75 nanoparticles on viability of melanoma (○) or on % sub-G1 nuclei from XPA cells (black bars on the right). The controls show the effect of cisplatin alone (●) or unloaded nanoparticles on cell viability (dashed line). $2x10^4$ cells were plated in 12 –well plates with 1 mL medium per well and allowed to grow as a subconfluent layer (24h /37 °C) before adding the nanoparticles or drug alone. The interaction between cells and nanoparticles took place for 48 h. Thereafter, XTT solution was added to evaluate the cell viability. The % sub-G1 nuclei was determined from flow cytometry.

In the CMC-Cis-CH75 nanoparticle, Cis is protected from rapid inactivation in the extracellular medium. Second, the release of cisplatin from the 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-acetyl-glucosaminidase [16]. Both enzymes are in the endosomal/ lysosomal vesicles so that the degradation and release of Cis will start immediately after endocytosis of the nanoparticles.

4 CONCLUSIONS

The biocompatible nanoparticles of CMC and CH75 employed in this work can effectively deliver cisplatin to cancer cells decreasing drug dose and improving cisplatin therapeutic index.

5 ACKNOWLEDGMENTS

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