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Preparation and Characterization of Cisplatin-Incorporated Chitosan Hydrogels, Microparticles, and Nanoparticles

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Abstract: Three different, polymer-platinum conjugates (hydrogels, microparticles, and nanoparticles) were synthesized by complexation of *cis*-dichlorodiammineplatinum(II) (cisplatin) with partially succinylated glycol chitosan (PSGC). Succinic anhydride was used as a linker to introduce cisplatin to glycol chitosan (GC). Succinylation of GC was investigated systematically as a function of the molar ratio of succinic anhydride to glucosamine, the methanol content in the reaction media, and the reaction temperature. By controlling the reaction conditions, water-soluble, partially water-soluble, and hydrogel-forming PSGCs were synthesized, and then conjugated with cisplatin. The complexation of cisplatin with water-soluble PSGC via a ligand exchange reaction of platinum from chloride to the carboxylates induced the formation of nano-sized aggregates in aqueous media. The hydrodynamic diameters of PSGC/cisplatin complex nano-aggregates, as determined by light scattering, were 180-300 nm and the critical aggregation concentrations (CACs), as determined by a fluorescence technique using pyrene as a probe, were 20-30 μg/mL. The conjugation of cisplatin with partially water-soluble PSGC, i.e., borderline between water-soluble and water-insoluble PSGC, produced micro-sized particles <500 μm. Cisplatin-complexed PSGC hydrogels were prepared from water-insoluble PSGCs. All of the cisplatin-incorporated, polymer matrices released platinum in a sustained manner without any significant initial burst, suggesting that they may all be useful as slow release systems for cisplatin. The release rate of platinum increased with the morphology changes from hydrogel through microparticle to nanoparticle systems.

Keywords: chitosan, platinum, hydrogel, microparticle, nanoparticle, controlled drug release.

Introduction

Cisplatin (*cis*-dichlorodiammineplatinum(II)), an anticancer drug, is used to treat various solid tumors, but it has a major drawback, namely non-specific toxicity, just as other chemotherapeutic agents. The clinical use of cisplatin is limited due mainly to serious side effects such as acute nephrotoxicity and chronic neurotoxicity.[1] To improve its therapeutic efficacy and reduce its non-specific toxicity, macromolecular drug delivery systems have been studied such as polymeric prodrugs,[2,3] micelles,[4-7] nanoparticles,[8] and microspheres.[9] The concept "the enhanced permeability and retention (EPR) effect" in tumor vasculature gave rise to extensive researches on macromolecular drug carriers.[10-13] It is generally accepted that macromolecules preferentially accumulated in tumor tissues than in normal tissues because of a unique feature of tumors greatly different from normal tissues, which is characterized by hypervasculature, defective vascular architec-

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ture, and impaired drainage systems. Some macromolecular prodrugs are progressing in clinical trials for cancer treatment.[12,14]

Chitosan, mainly composed of 2-amino-2-deoxy-β-D-glucopyranose (D-gluocosamine), is one of the most abundant natural polysaccharides. Chitosan meets two important requirements as a biomaterial, biocompatibility and biodegradability. Increasing attention has been paid to its biomedical applications such as drug delivery systems,[15-20] gene delivery systems,[21-25] artificial skins,[26] and tissue engineering.[27-29] Indeed, chitosan is a versatile carrier for drug and gene deliveries. It has reactive amino groups at the C2 position of glucosamine, which can be readily used for chemical modification, conjugation with drugs and cell/tissue-targeting moieties, and complexation with oligonucleotides, siRNA, and DNA.

Recently, our group developed a novel type of chitosan-based polymeric amphiphile, composed of hydrophilic glycol chitosan (GC) and hydrophobic doxorubicin.[30] The glycol chitosan-doxorubicin (GC-DOX) conjugates spontaneously formed compact nano-sized self-aggregates in an aqueous medium. When systemically administrated into tumor-bearing rats, the GC-DOX self-aggregates accumulated in tumor as a function of time, thus suppressing the tumor growth and significantly reducing side effects of doxorubicin. The chitosan nano-aggregates exhibited much more prolonged systemic retention than common macromolecules, even polymeric micelles.

Herein we report the preparation and characterization of three different chitosan-based formulations (hydrogels, microparticles, and nanoparticles) incorporating cisplatin. These formulations have been developed by complexation of cisplatin with partially succinylated glycol chitosans (PSGCs) showing different aqueous solubilities. The reaction conditions to give PSGCs with different physicochemical characteristics were systematically investigated. The platinum release profiles from the formulations were studied in PBS. Additionally, the colloidal characteristics of the nanoparticles prepared by complexation of cisplatin with water-soluble PSGC were investigated by dynamic light scattering and fluorescence spectroscopy.

Experimental

Materials. Cisplatin (purity 99.9%) and succinic anhydride were purchased from Sigma (St. Louis, MO, USA). Glycol chitosan (GC, MW 250 kDa, degree of deacetylation 88.7%), purchased from Sigma was dissolved in distilled water, filtered to remove insoluble impurities, and dialyzed against distilled water. All other chemicals were of analytical grade and were used as received.

Synthesis and Characterization of PSGC. GC was acylated with succinic anhydride. GC (0.2 g) was dissolved in distilled water (10 mL). Different amounts of methanol were added into the aqueous GC solution and stirred for 1 h. The desired amount of succinic anhydride in methanol (10 mL) was added in the methanolic GC solution and stirred for 24 h. The reaction mixture was dialyzed against distilled water for 2 days and lyophilized. Degree of succinylation (DS) was determined by ^{1}H NMR spectroscopy and elemental analysis. ^{1}H NMR spectra were recorded on UnityPlus 300 (Varian, Inc. Palo Alto, CA, USA), which was operated at 300 MHz. The samples were dissolved in D_2O/CF_3COOD to give a polymer concentration of 1% (w/v). The chemical shifts were expressed in ppm, based on the signal for sodium 3-(trimethylsilyl)-propionate-d_4 used as an internal reference. Element analysis was performed with EA1110 (CE instrument, Lakewood, NJ, USA). DS was calculated from C/N ratio.

Complexation of Cisplatin with PSGC. Cisplatin and PSGCs were separately dissolved (or suspended) in distilled water. Two solutions were mixed and stirred for 24 h at room temperature, and neutralized with 0.02 M NaOH. The resultant solution was dialyzed against distilled water for 2 days, and lyophilized. The cisplatin content in the complex was determined with an inductively-coupled plasma (ICP)-atomic emission spectrometer (ICPS-100IV, Shimadzu Corp., Kyoto, Japan) calibrated with a standard cisplatin solution.

Preparation and Characterization of PSGC/Cisplatin Complex Nanoparticles. The cisplatin/PSGC complexes were suspended in distilled water under gentle shaking, followed by sonication using a probe-type sonicator (Ultrasonic Processor GEX-600, Sigma) at 60 W for 1 min, in which the pulse was turned off for 1 sec with the interval of 5 sec. The nanoparticle solution was passed through a syringe filter (pore size 0.45 μm, Millipore, Billerica, MA, USA). To determine the particle size and size distribution of PSGC/cisplatin complex nanoparticles, dynamic light scattering (DLS) measurements were performed using a helium ion laser system (Spectra Physics Laser Model 127-35, Mountain View, CA, USA), which was operated at 633 nm and 25±1 °C. The intensity autocorrelation was measured at a scattering angle (θ) of 90° with a Brookhaven BI-9000AT autocorrelator (Holtsville, NY, USA) at 25 °C. When the difference between the measured and the calculated baseline was less than 0.1%, the correlation function was accepted. CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain size distribution of nanoparticles. The mean diameter (d) was evaluated from the Stokes-Einstein equation. Critical aggregation concentration (CAC) was determined by a fluorescence technique using pyrene as a probe. Steady-state fluorescence spectra were recorded on a RF-5301 fluorometer (Shimadzu Corp., Kyoto, Japan). A sample solution containing pyrene (1.2×10^{-7} M) was excited using an 150 W xenon arc lamp (ILC Technology, Sunnyvale, CA, USA). The conjugate concentration varied from 1 to 500 μg/mL. For the measurement of pyrene emission spectra, the slit openings for excitation and emission were set

at 2.0 and 0.5 mm, respectively. The excitation wavelength was 336 nm.

In vitro Release of Platinum from PSGC/Cisplatin Complexes. In vitro release profiles of platinum from PSGC/cisplatin complexes were examined at 37 °C in PBS. The freeze-dried PSGC/cisplatin complexes were weighed and dissolved (or suspended) in distilled water (10 mL), and introduced into a dialysis membrane bag (MWCO 1,500 Da, Spectrum Lab., Rancho Dominguez, CA, USA). The release experiments were initiated by placing the end-sealed dialysis bags in distilled water (100 mL) and incubations were carried out in a water bath at 37 °C under gentle shaking. Periodically, samples (10 mL) were withdrawn and replaced with an equal volume of fresh medium. The amount of released platinum was determined using an ICP-atomic emission spectrometer.

Results and Disscussion

Succinylation of GC. Succinyl groups were introduced to GC for coupling with cisplatin through chelate-type coordination bonds to give a macromolecular prodrug of cisplatin. GC was acylated with succinic anhydride under different reaction conditions. Five molar ratios of succinic anhydride to glucosamine unit, 3 methanol contents in reaction media, and 3 reaction temperatures were used as variables. Degree of succinylation (DS) of PSGC was determined from ^{1}H NMR spectroscopy and elementary analysis. Figures 1 and 2 show ^{1}H NMR spectra of PSGCs. The sample code, T*a*M*b*R*c*, represents the PSGC which was synthesized at *a* reaction temperature (°C), *b* methanol content (% (v/v)) in reaction media and *c* molar ratio of succinic anhydride to glucosamine of GC. The peaks of *N*-succinic protons were observed at 2.48 (-NHCOC**H**$_2$-) and 2.62 (-C**H**$_2$COOH) ppm, as shown in Figure 1. The peaks of O-succinic protons were observed at 2.56 ppm, as shown in Figure 2. The peak at 2.98 ppm was assigned to C2 proton of glucosamine unit. DS was calculated from the integrated peak areas of succinic protons and C2 proton. Table I shows DS determined by ^{1}H NMR spectroscopy. DS by elementary analysis (data not shown) was not significantly different from that by ^{1}H NMR spectroscopy.

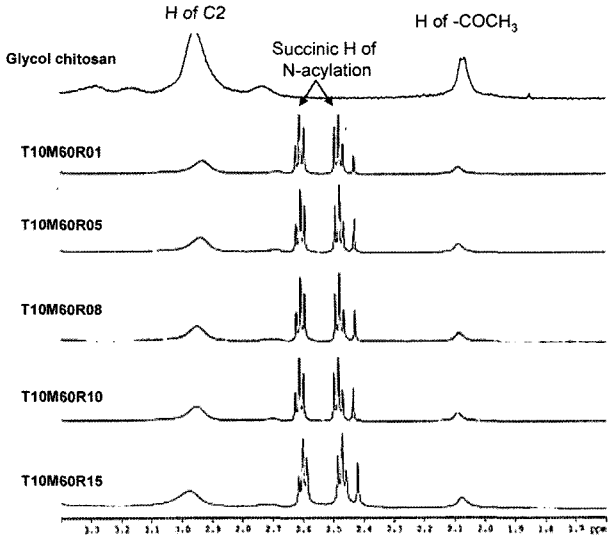

Figure 1. ^{1}H NMR spectra of T10M60 series. T*a*M*b*R*c* represents the PSGC which was synthesized at *a* reaction temperature (°C), *b* methanol content (% (v/v)) in reaction media, *c* molar ratio of succinic anhydride to glucosamine of GC.

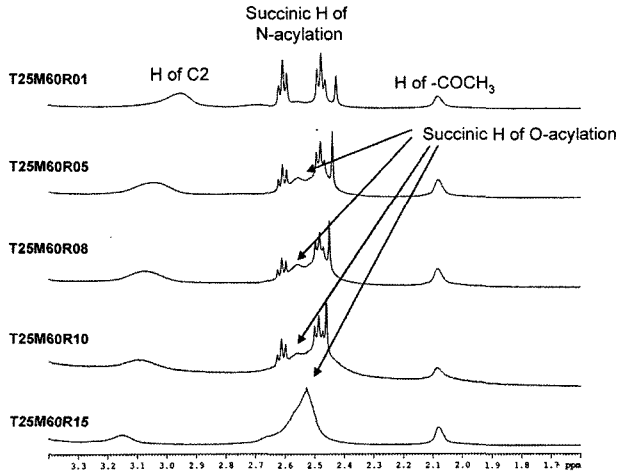

Figure 2. ^{1}H NMR spectra of T25M60 series. T*a*M*b*R*c* represents the PSGC which was synthesized at *a* reaction temperature (°C), *b* methanol content (% (v/v)) in reaction media, *c* molar ratio of succinic anhydride to glucosamine of GC.

The addition of excessive anhydride was not effective on increasing DS. Just a slight increase in DS was observed. The low reaction temperature (10 °C) was favorable to the selective *N*-succinlyation, as shown in Figure 1. The PSGCs synthesized at 10 °C (T10 series) were all freely water-soluble. In the specific amount of methanol content (60% (v/v)) and high molar ratios of succinic anhydride at 25 °C, *O*-succinylation was predominantly occurred, as shown in Figure 2. The *O*-succinylated GCs showed low aqueous solubility. T25M60R05 (*-marked in Table I) showed partial water-solubility and formed a milky solution during dialysis. From T25M60R08 to T25M60R15 (gray-colored in Table I), they were water-insoluble and formed hydrogels during dialysis, and were not redissolved in water after gelation. T50M60R10, T50M60R15, and T50M66R15 (gray-colored in Table I) formed hydrogels during dialysis, as well.

PSGC/Cisplatin Complexes. PSGC/cisplatin complexes were prepared through the ligand exchange reaction of cisplatin from the chloride to the carboxylate groups of PSGC in an aqueous medium. Two water-soluble PSGCs, T10M66R08, and T50M60R05, were complexed with cisplatin. Their sample codes are Pt1-T10M66R08, Pt2-T10M66R08, Pt1-

Table I. Degrees of Succinylation (DS) of PSGCs Calculated from ^{1}H NMR

T^a(°C)	MeOH^b \ MR^c	1	5	8	10	15
10	50	-	0.40	0.44	0.42	0.42
	60	0.43	0.40	0.41	0.41	0.40
	66	0.40	0.37	0.46	0.42	-
25	50	0.35	0.31	0.32	0.35	0.49
	60	0.37	*0.43	0.42	0.36	1.11
	66	0.38	0.38	0.35	0.37	0.36
50	50	0.32	0.34	0.35	0.36	0.35
	60	0.37	0.34	0.42	0.41	0.62
	66	0.36	0.36	0.34	0.37	0.45

^{a}T: Reaction temperature. ^{b}MeOH: Methanol content (% (v/v)) in reaction media. ^{c}MR: Molar ratio of succinic anhydride to glucosamine unit. Gray-colored: Formed Hydrogels. *A translucent milky solution.

T50M60R05, and Pt2-T50M60R05. In addition, one (T25M60R10) of hydrogel samples and one (T25M60R05) of partially water-soluble samples were complexed with cisplatin. Their sample codes are Pt1-T25M60R10, Pt2-T25M60R10, Pt1-T25M60R05, and Pt2-T25M60R05. Table II shows cisplatin contents of PSGC/cisplatin complex conjugates measured by ICP-atomic emission spectroscopy. The cisplatin contents in the conjugates were in the range of 2.2-5.7% (w/w).

The samples from water-soluble PSGCs (T10M66R08 and T50M60R05) formed nano-sized aggregates after complexation with cisplatin. The sample from partially water-soluble GC (T25M60R05) gave milky solutions with microparticles (<500 μm) after complexation with cisplatin. Cisplatin-complexed PSGC hydrogels were prepared from the water-insoluble PSGC sample (T25M60R10).

As described above, the conjugation of cisplatin with the freely water-soluble PSGCs (T10M66R08 and T50M60R05) induced the spontaneous formation of nano-sized aggregates in aqueous milieu. Figure 3 shows particle size and size distribution of PSGC/cisplatin complex nano-aggregates (Pt1-T10M66R08) measured by dynamic light scattering. Kataoka *et al.* reported a series of polymer-cisplatin complex micelles.[4-7] In their works, a simple mixing of cisplatin with a block copolymer, poly(ethylene glycol)-*b*-poly(α,β-aspartic acid) (PEG-P(Asp)) in aqueous media led to a spontaneous micelle formation. The cisplatin-complexed micelles had diameters of 20 nm with narrow size distribution. As compared with the complex micelles by Kataoka *et al.*, the PSGC/cisplatin complex aggregates had much larger particle sizes. Their mean diameters were in the range of 180 to 300 nm. While the flexible, linear block copolymer PEG-P(Asp) formed a typical polymeric micelle by complexation with cisplatin, and it had relatively small size ~20 nm and a core-shell structure, the PSGC which has a rigid and bulky

Table II. Cisplatin Contents of PSGC/Cisplatin Complexes Measured by ICP-Atomic Emission Spectroscopy

Sample Code	Sample Description	Cisplatin Content (% w/w)
Pt1-T10M66R08	Nano-aggregates	2.54
Pt2-T10M66R08		2.80
Pt1-T50M60R05		2.81
Pt2-T50M60R05		3.80
Pt1-T25M60R05	Milky Solutions	2.26
Pt2-T25M60R05		5.30
Pt1-T25M60R10	Hydrogels	2.88
Pt2-T25M60R10		5.70

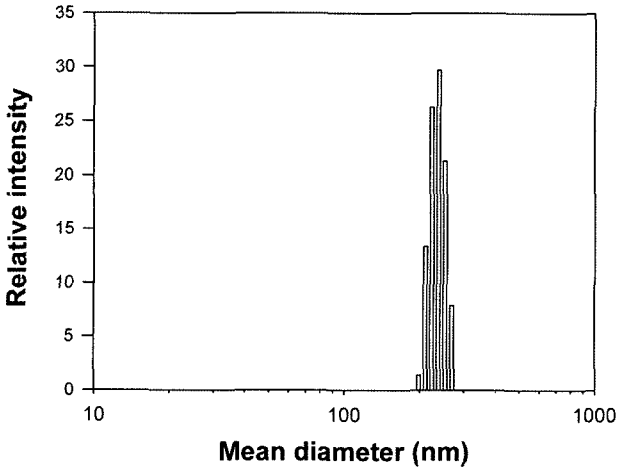

Figure 3. Particle size and size distribution of PSGC/cisplatin complex nano-aggregates (Pt1-T10M66R08) in distilled water measured by dynamic light scattering.

polysaccharide backbone formed a relatively large and coarse aggregate through complexation with cisplatin.

The nanoparticle formation of PSGC/cisplatin complexes in aqueous media was monitored by a fluorescence technique using pyrene as a probe.[31-34] Figure 4 shows the effect of polymer concentration on the fluorescence emission spectra of pyrene. Pyrene is sparingly soluble in water, and in a system composed of both hydrophobic and hydrophilic phases, it is preferentially partitioned in the hydrophobic phase and strongly emits, which results in dramatic increase of the third highest vibrational band at 393 nm. Figure 5 shows the intensity ratio (I_1/I_3) of the first and third highest energy bands in the emission spectra of pyrene in PSGC/cisplatin nano-aggregate solutions. At a low concentration of the conjugate, a negligible change in the intensity ratio was observed, but over a critical concentration, the intensity ratio decreased linearly with the addition of the conjugate. This threshold concentration is defined as a critical aggregation concentration (CAC), which can be determined from the crossover point of two straight lines, as shown in Figure 5. The CACs of PSGC/cisplatin nano-aggregates were 20-30 μg/mL, which are comparable to those of self-aggregates of hydrophobically modified chitosans (19-66 μg/mL) and much lower than the critical micelle concentration (CMC, 2.3 mg/mL) of a low molecular surfactant, sodium dodecyl sulfate (SDS).[32] The low CAC suggests that the PSGC/cisplatin complex nano-aggregates have a good colloidal stability in an aqueous phase as do self-aggregates of hydrophobically modified chitosans.

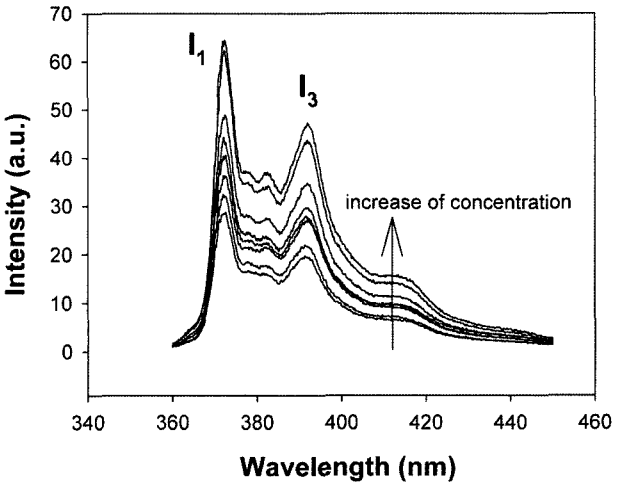

Figure 4. Fluorescence emission spectra of pyrene (1.2×10^{-7} M) as a function of Pt1-T50M60R05 concentration. The excitation wavelength was 336 nm. The polymer concentration varied from 1 to 500 μg/mL.

In vitro Release of Platinum from Hydrogels, Microparticles, and Nanoparticles. The chelate-type coordination bond between platinum and carboxylic group is known to be stable in pure water, however, undergoes ligand substitution with various ions, leading to release of platinum.[4-7] As expected, the cisplatin-incorporated PSGC hydrogels, microparticles, and nano-aggregates were stable in distilled water for a prolonged period of time; e.g. the amount of platinum released from nano-aggregates for 10 days were below 5% in distilled water. The in vitro release profiles of cisplatin from three different types of PSGC matrices (hydrogels,

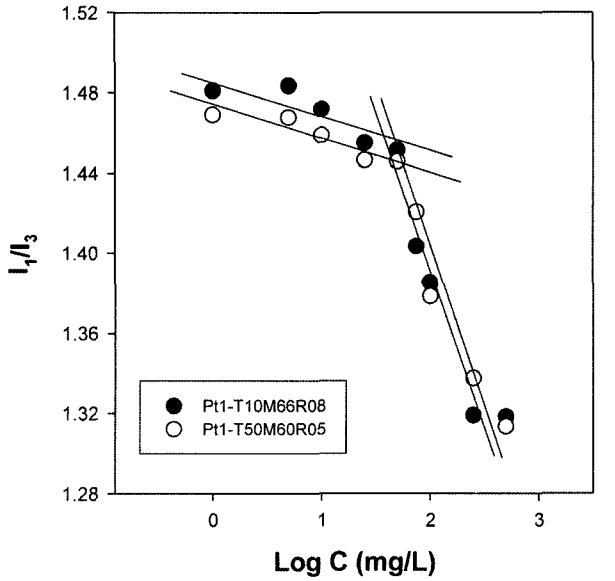

Figure 5. Intensity ratio (I_1/I_3) for pyrene as a function of Pt1-T50M60R05 concentration in distilled water. The excitation wavelength was 336 nm.

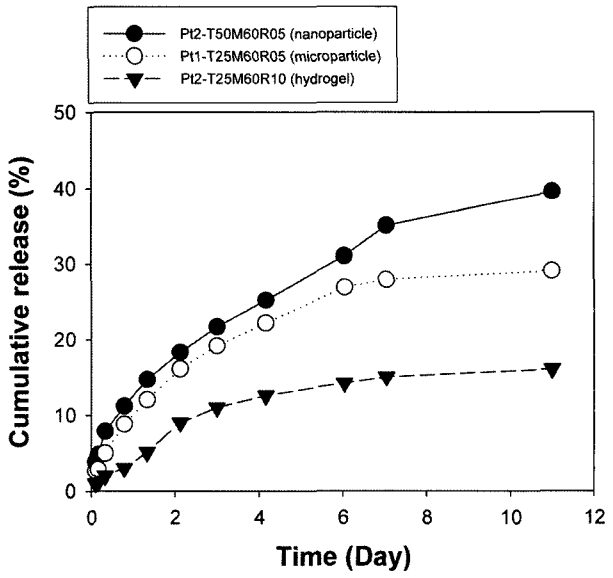

Figure 6. The in vitro release profiles of platinum from three different types of PSGC/cisplatin complex matrices (hydrogels, microparticles, and nano-aggregates) in PBS at 37 °C.

microparticles, and nano-aggregates) in PBS at 37 °C are shown in Figure 6. All of the cisplatin-incorporated matrices released platinum in a sustained manner in PBS, and no significant initial burst was observed. In chloride ion-rich solutions like PBS, the carboxylic ligand is a good leaving group and reversible to the chloride ligand due to an inverse ligand substitution of the Pt(II) atom from PSGC to chloride, which results in release of platinum from the polymer matrices. The release rate of platinum was the highest in the nano-aggregate system, the lowest in the hydrogel system. Presumably, the hydrogel may have more compact inner structure, which acts as a barrier against free ion exchange. All three cisplatin-incorporated formulations appeared to be useful as slow release systems for cisplatin.

Conclusions

PSGCs with different aqueous solubilities such as water-insoluble, partially water-soluble and water-soluble were prepared by succinylation of GC. The aqueous solubility of PSGC mainly depended on regioselective succinylation. The *N*-succinylated GC exhibited good water-solubility, while *O*-succinylated GC showed poor water-solubility. The complexation of cisplatin with PSGCs provided cisplatin-incorporated hydrogels, microparticles, and nanoparticles depending on the aqueous solubility. Notably, the complexation of cisplatin with water-soluble PSGCs led to nano-aggregates with mean diameters of 180-300 nm. Their CACs measured by a fluorescence technique were quite low, 20-30 μg/mL, indicating the formation of stable nano-aggregates in aqueous media. The cisplatin-incorporated hydrogels, microparticles, and nanoparticles released platinum in a sustained manner for a prolonged period of time in chloride-rich, aqueous solutions.

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