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Preliminary toxicological studies of selected water-soluble polymer–platinum conjugates

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The objective of this preliminary investigation of a number of water-soluble carrier-bound platinum(II) complexes for potential use in cancer chemotherapy was to assess the toxicological behavior of representative platinum coordination compounds anchored to, or incorporated into, polymeric carriers via polymer-attached amine ligands. The conjugates included linear polyaspartamides (1–4, 6, 7), each composed of a major fraction of subunits featuring side-chain-attached tertiary amino groups as water-solubilizing entities, and a minor fraction of subunits comprising the anchored platinum complexes, again as side-chain components. Whereas in 1–4 the platinum atom was polymer-bound through a single amino group, both 6 and 7 contained polymer-attached *cis*-diamine-chelating ligands coordinating to the metal center. Also included in this study was a linear polyamidoamine (5), which contained a poly(ethylene oxide) segment in the backbone in addition to intrachain ethylenediamine segments acting as *cis*-diamine chelating ligands for coordination to the platinum center. The compounds were injected as aqueous (phosphate-buffered saline) solutions into the tail veins of CD-1 mice (four to eight mice per conjugate), and the maximally tolerated dose was determined for each compound. For polyaspartamides 1–4 the dose levels ranged from about 25 mg Pt (kg body weight^{-1}) (in conjugate 4) to 500 mg Pt kg^{-1} (in compound 1), the latter conjugate proving some 100-fold less toxic than cisplatin (3–4 mg Pt kg^{-1}), which was included in this study for comparison. Low toxicity (tolerated dose 160 mg Pt kg^{-1}) was also observed for the intrachain *cis*-diamineplatinum complex polymer (5). The polyaspartamide conjugates 6 and 7, on the other hand, both characterized by a *cis*-diamineplatinum complex system in the side chain, were toxic even below the dose level of 20–25 mg Pt kg^{-1}. The preliminary findings of this study, while providing a basis for more extensive and broad-based toxicological studies, will serve to direct and optimize structural conjugate designs in forthcoming synthetic programs.

Keywords: platinum complexes; cancer chemotherapy; polyaspartamide; polyamidoamine; toxicology; water-soluble; carrier-bound

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INTRODUCTION

In a series of preceding communications the synthesis of water-soluble and biodegradable polymer–platinum conjugates was described in which monoamine- and *cis*-diamine-platinum complexes related to the anticancer drug cisplatin [*cis*-diamminedichloroplatinum(II)] were covalently attached (conjugated) to linear, macromolecular carrier molecules.[1–6] The polymer-drug conjugates, constructed in accordance with established biomedical design requirements, were intended to serve as potential prodrugs in cancer chemotherapy, the rationale being that improved pharmacokinetic efficacy should lead to selective accumulation in cancer tissue and facilitated endocytotic cell entry. Enzymic or hydrolytic action on the conjugate in the lysosomal compartment should then cause liberation of the active platinum complex from the carrier for ultimate interaction with the nuclear DNA of the affected target cells, and this should

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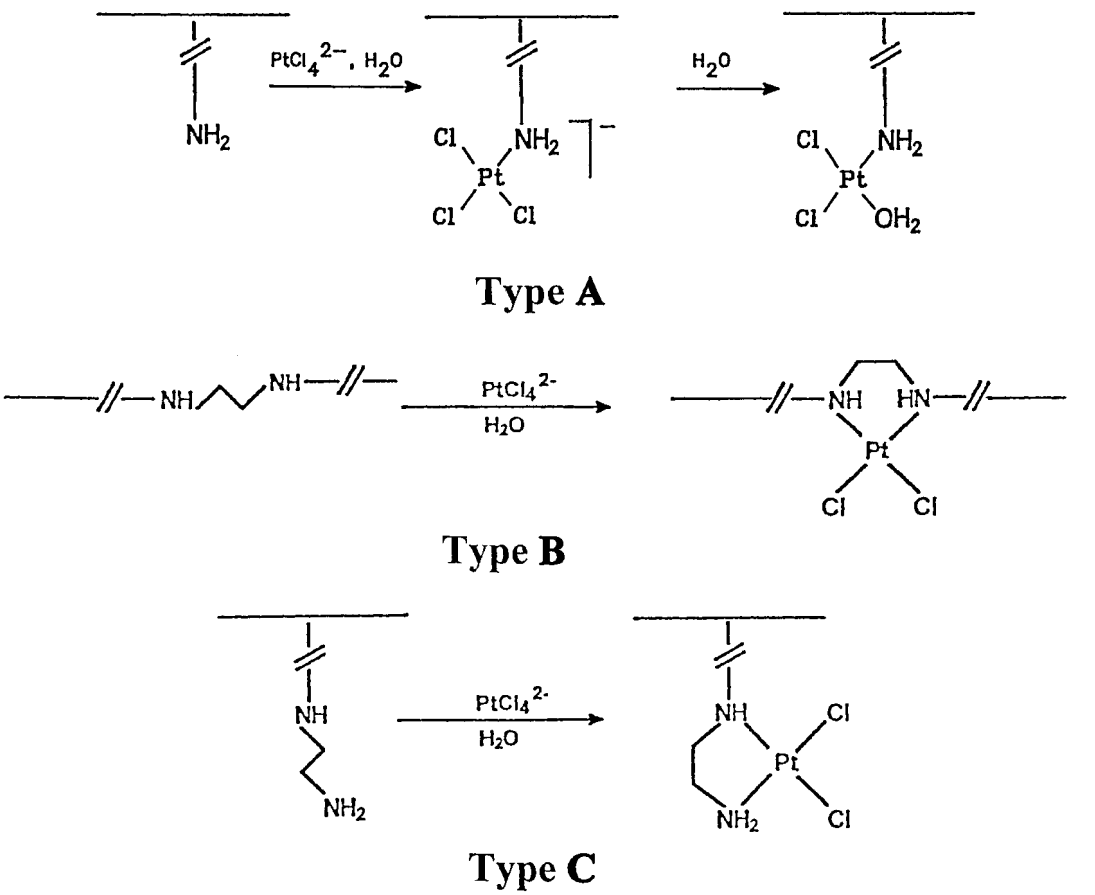


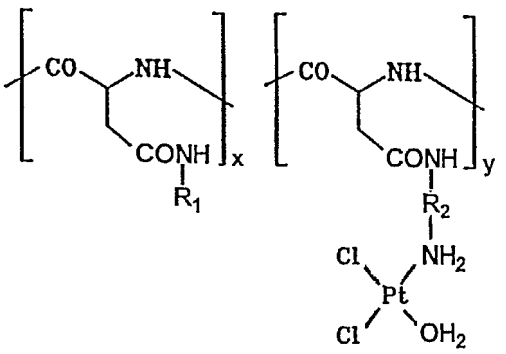
Scheme 1 The three structural types of conjugate studied in this work.

result overall in reduced toxicity and increased bioavailability and concomitantly enhanced therapeutic effectiveness. Selected members of these variably constructed platinum conjugates were recently tested *in vitro* for antiproliferative activity against human cervical carcinoma (HeLa) cells[7,8] and other cell lines[9] with encouraging results. To arrive at a preliminary assessment of the toxicological behavior of the synthesized polymers, we have determined, and report here, the tolerated dose levels *in vivo* for some representative polymer–platinum conjugates developed in the preceding investigations.

RESULTS AND DISCUSSION

Three different structural types of conjugate, schematically depicted by **A, B** and **C** (Scheme 1), were studied. In **A** the platinum atom is polymer-anchored through a single, carrier-attached amine ligand, and we ascribe the aquadichloroplatinum(II) structure to the conjugate complex. **B** represents a conjugate model in which the metal is coordinated by two *cis*-oriented, secondary intrachain amino groups, and the active complex therefore constitutes a main-chain component. A *cis*-diamine–metal coordination pattern is also present in **C**, although the amino groups, primary and secondary in this scheme, are constituents of polymer-attached side chains.

Specifically, the conjugates investigated, all completely soluble in water, possessed the structures **1–7** (Scheme 2) and were prepared by previously elaborated, slightly modified procedures.[1–7] Conjugates **1–4**, all of Type **A**, were polyaspartamides distinguished from one another by the nature of the hydrosolubilizing side groups, 3-(dimethylamino)propyl in **1** and **3**, and 3-

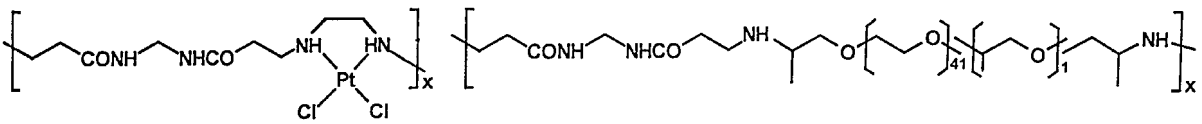


1: R_1 = NMe$_2$ R_2 = x/y = 3

2: R_1 = N O R_2 = x/y = 3

3: R_1 = NMe$_2$ R_2 = O O x/y = 9

4: R_1 = N O R_2 = O O x/y = 9



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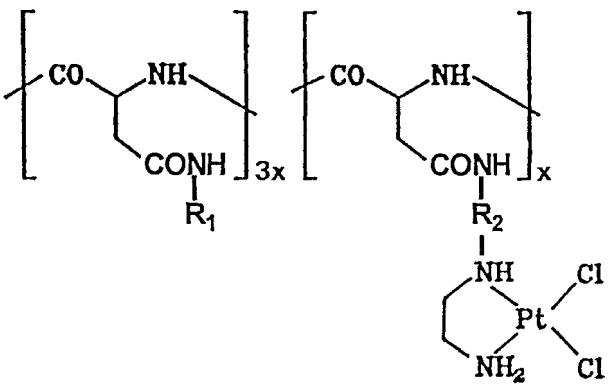

6: R_1 = NMe$_2$ R_2 =

7: R_1 = N O R_2 =

Scheme 2 Structural representations of the conjugates **1–7** (protonation effects are ignored).

Table 1 Maximum tolerated dose levels[a] for **1–7**

Conjugate	Number of mice per sample	Nominal Pt content (%)[b]	Max. tolerated dose	
			mg polymer kg^{-1}	mg Pt kg^{-1}
1	8	18.5	2800	518
2	5	16.5	900	145
3	8	8.4	500	42
4	9	7.2	375	27
5	8	7.4	2000	160
6	5	18.3	<150	<27
7	4	16.4	<150	<25
Cisplatin	2	65.0	4.6[c]	3–4

[a] See the Experimental section for procedure.
[b] Mass percentage of platinum in unprotonated compounds (see the Experimental section for compositions).
[c] Given as mg cisplatin kg^{-1}. In other investigations (Refs 14, 15), we found 7.5 mg cisplatin kg^{-1} to be toxic, 4 mg kg^{-1} to be non-toxic (Ref 16.), and 5 mg kg^{-1} to be borderline, causing 40% death in one study (Ref 14.) but no death in another (Ref 15.).

(morpholin-4-yl)propyl in **2** and **4**, and also by the nature of the carrier–ligand spacers, a propylene segment in **1** and **2**, and a 1,2-bis(ethyleneoxy)-ethane segment in **3** and **4**. Release of the aquated monoamineplatinum(II) complex system as the ultimately active agent requires lysosomal cleavage of the side-chain amide group (symbolized in Scheme 1 by //). Polyamidoamine **5**, an intrachain polymer complex of Type **B**, must undergo complete backbone fission at the cleavage sites // for liberation of the monomeric, aquated *cis*-diamineplatinum(II) moiety, a process expected to be considerably retarded relative to that in Type **C**. Representatives of Type **C** are the polyaspartamides **6** and **7**, which again are differentiated by the nature of the hydrosolubilizing group. Release of the active aquated *cis*-diamineplatinum complex will require fission of the side-chain amide group, as in Type **A** (Scheme 1).

For the toxicological work, the polymer samples were dissolved in phosphate buffer at typical concentrations of 10–100 mg of polymer per ml. The mice (CD-1, male) were weighed, and increasing doses in 0.1–0.5 ml aliquots of the filtered solutions were injected into their tail veins. The body weight and general appearance of the animals so treated were monitored over a period of 20 days post-injection. Animals alive and well by day 20 were considered to be long-term survivors, and the maximal dose level administered to allow for long-term survival of, for example, two mice out of two animals injected was taken as the maximum tolerated dose for the conjugate in question. The dose levels were established for **1–7** in terms of mg of polymer per kg body weight and ultimately, as a common base of comparison, were expressed in units of mg Pt kg^{-1}. The data are listed in Table 1. Graphical representations for the first two conjugates of the series (Fig. 1) illustrate the general trend.

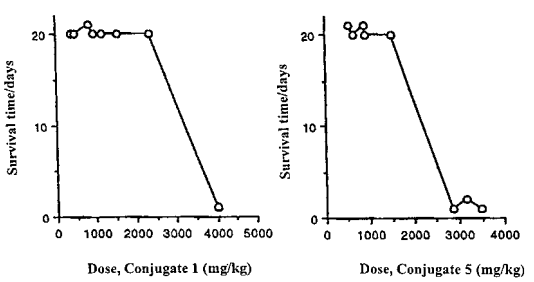

Figure 1 Survival time versus dose for conjugates **1** and **5**.

Conjugate **1**, with a dose level of >500 mg Pt kg^{-1}, was the most readily tolerated, and hence least toxic, compound (Table 1). Set against cisplatin, for which we redetermined a maximum tolerated dose of 3–4 mg Pt kg^{-1}, the conjugate proved less toxic by a factor greater than 100. For the analogous **2**, with an aminopropylmorpholine in place of an aminopropyldimethylamine hydrosolubilizing side group, the tolerated dose, 145 mg Pt kg^{-1}, is lower, although still nearly 50 times higher than for cisplatin. Substantially lower doses, 42 and 27 mg Pt kg^{-1}, respectively, were determined for the closely related conjugate pair **3** and **4**, and at present we have no reasonable explanation for this unexpected difference. Once again, the aminopropylmorpholine-containing **4** was found to be more toxic than the dimethylaminopropyl-containing analogue **3**. It will be of interest in future work to examine the toxicological features of

the metal-free carriers with a view to establishing differences, if any, in the effects exerted by the two dissimilar hydrosolubilizing groups, and equally so, by the spacers tying the metal-coordinating amine ligand to the polymer main chain.

An interesting picture, lastly, emerges from a comparison of the toxicological features of the three conjugates **5–7** characterized by a platinum bonding pattern in which the metal is polymer-anchored via the *cis*-diamine ligand system. Whereas the poly(ethylene oxide)-modified polyamidoamine **5** allows for a remarkably large tolerated dose (160 mg Pt kg^{-1}), both polyaspartamides **6** and **7** were found to be lethally toxic even at the lowest dose level tested, 150 mg of polymer per kg, which translates into distinctly less than 25 mg Pt kg^{-1} of tolerated dose. In light of the moderately or appreciably better tolerance provided by the conjugates **1–4**, the observations regarding **6** and **7** are not convincing; the toxicities may have resulted from factors unconnected with the platinum complex structure, such as coagulation or clotting in central circulation. These preliminary findings, therefore, require confirmation in a more extended testing program involving sets of identical conjugates prepared separately in independent experiments. The present data will serve to develop strategies for the synthesis of carrier polymers comprising backbone and side-chain structures modified for enhanced *in vivo* tolerance of derived conjugates.

EXPERIMENTAL

General procedures

Solid-state IR spectra (KBr pellets) were recorded over the region 4000–200 cm^{-1}. ^{1}H NMR spectra (400 MHz) were taken in D_2O solutions; chemical shifts, δ, are given in ppm relative to internal sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (integration error limits ±12%). Immediately before recording, the pH of the solutions was adjusted to 10 (KOH) in order to eliminate potential protonation effects. Cannon–Fenske tubes were used for the determination of inherent viscosities, η_{inh}, in deionized water at 30.0 ± 0.5 °C; the concentration was c = 0.2 g/100 ml; the findings are given in units of ml g^{-1}. Spectra/Por 4 membrane tubing (Spectrum Industries, Los Angeles, CA, USA), with molecular mass cut-off limits of 12 000–14 000, was used routinely for dialysis of carriers and conjugates. The carrier polymers were additionally dialyzed in Spectra/Por 6 wet tubing with cut-off limit 25 000. Freeze-drying operations were carried out with the aid of a Virtis Bench Top 3 freeze-drier at −30 °C, 0.1 Torr. Platinum-free polymers were routinely post-dried in a Sartorius Thermo Control Infrared Drying System (heating program 2 × 8 min at 65 °C); alternatively, an Abderhalden drying tube was used (two days at 60 °C, 10–20 Torr). The Abderhalden equipment was also employed for a post-drying operation (two days at 65 °C, 10 Torr) of sample material prepared for microanalysis. Platinum determinations (in duplicate; data averaged) were made in the Anglo American Research Laboratories, Crown Mines, Johannesburg.

Solvents, reagents and reactants

Deionized water was used for preparative and dialysis operations. *N*,*N*-Dimethylformamide (DMF), predried over molecular sieves, 4A, was redistilled under reduced pressure in a faint stream of N_2. All other solvents, laboratory grade, were used as received. Potassium tetrachloroplatinate(II) (Strem Chemicals) and other reactants, reagent grade (Fluka Chemie), were used as received. These included the poly(ethylene glycol) derivative, Jeffamine ED 2001 (nominal molecular mass 2000), designated by the supplier as *O*,*O*′-bis(2-aminopropyl) poly(ethylene glycol) 1900. ^{1}H NMR data have shown this compound to possess the average composition:

$$H_2NCH(CH_3)CH_2O[CH_2CH_2O]_{41}[CH(CH_3)CH_2O]_1CH_2CH(CH_3)NH_2$$

with M = 1996.

Polymeric carriers and conjugates

Poly-D,L-succinimide, the educt in the syntheses of the polyaspartamide carriers as precursors to conjugates **1–4**, **6** and **7**, was prepared by a literature method;[9] a mass-average molecular mass of 36 700 was determined from viscometric data.[10] The polymer was redried in a Sartorius IR heating system before use. Amounts of polymeric educts and products are given as base moles, i.e. moles of recurring units normalized to x = 1 (y = 1 in **1–4**).

Conjugate 1

The carrier component of this conjugate, poly-α,β-D,L-[*N*-(3-dimethylaminopropyl)aspartamide(75)-

co-*N*-(3-aminopropyl)aspartamide(25)], synthesized by a previously described procedure[11] [for which it was designated **11**(75)], was additionally dialyzed in Spectra/Por 6 wet tubing for two days, freeze-dried and post-dried. ^{1}H NMR data were in accord with those described previously.[11] For conversion of the carrier to the conjugate, the platination method described earlier[2] was used, with minor modification. A 386-mg portion (0.5 mmol) of the carrier was dissolved in 10 ml of H_2O. K_2PtCl_4, (250 mg; 0.6 mmol), was added and dissolved, while a slow stream of N_2 was introduced to saturation. The orange–red solution was stirred in a stoppered flask with protection from light for 24 h at ambient temperature and for another 20 h at 55 °C. Throughout the heating period, the pH was maintained at 5–6, and only for the last 20 min was it lowered to 4–5 (HCl). The routinely filtered solution, upon addition of NaCl (0.5 g), was stirred for 3 h at room temperature and dialyzed in Spectra/Por 4 tubing for 2 h against H_2O at pH 7 and for another 50 h against several batches of H_2O acidified to pH 6 and ultimately pH 5 (HCl). Freeze-drying of the retentate gave 364 mg (69.2%) of light-brown, water-soluble solid; η_{inh}, 15 ml g^{-1}. Analysis: Found: Pt, 19.1. Calcd for $(C_{34}H_{66}Cl_2N_{12}O_9Pt)_n$ (**1**) $(1052.9)_n$: Pt, 18.5%. Protonation effects were neglected in this and subsequent conjugate compositions.

Conjugate 2

By the procedure described for the foregoing experiment, this conjugate was prepared in 63% yield from the polyaspartamide carrier, poly-α,β-D,L-[*N*-(3-(morpholin-4-yl)propyl)aspartamide-(75)-*co*-*N*-(3-aminopropyl)aspartamide(25)], designated **5**(75) in the previous paper,[11] as a tan-brown, water-soluble solid; η_{inh}, 14 ml g^{-1}. Analysis: Found: Pt, 16.1. Calcd for $(C_{40}H_{72}Cl_2N_{12}O_{12}Pt)_n$ $(1179.0)_n$ (**2**): Pt, 16.5%.

Conjugate 3

The carrier component of this conjugate, poly-α,β-D,L-[*N*-(3-dimethylaminopropyl)aspartamide(90)-*co*-*N*-(9-aza-3,6-dioxanonyl)aspartamide(10)], was synthesized, with some modification, by a recently described procedure.[12] To the stirred solution of polysuccinimide, 1.94 g (20 mmol), in 40 ml of dimethylformomide (DMF), was added 3-(dimethylamino)propylamine (1.84 g; 18 mmol), dissolved in 10 ml of DMF. After saturation with N_2, the solution was stirred in a stoppered flask for 20 h at room temperature and cooled in an ice bath. Ethylenedioxy-*O*,*O*′-bis(2-ethylamine) (889 mg; 6 mmol), dissolved in 15 ml of DMF, was added rapidly with renewed nitrogen saturation, and the solution was stirred for 24 h at ice-bath temperature and another 60 h at ambient temperature. Up to this point, moisture access was rigorously precluded in an effort to prevent hydrolytic imide ring-opening in the intermediate polymer. Volume reduction to *ca* 15 ml by rotary evaporation was followed by product precipitation with 50 ml of Et_2O–hexane (2:1, v/v). The precipitate was thoroughly washed with hexane and warm acetone for removal of admixed ethylenedioxybisethylamine and, upon dissolution in 20 ml of H_2O, was dialyzed successively for 48 h in Spectra/Por 4, and another 48 h in Spectra/Por 6 tubing. Freeze-drying of the retentate and post-drying in the Sartorius system afforded the polyaspartamide as an off-white, water-soluble solid in a yield of 2.53 g (62.1%); η_{inh}, 14 ml g^{-1}. The ^{1}H NMR spectrum was substantially identical to that of the previously prepared[12] compound. The carrier was platinated essentially by the method described for **1**; however, the heating conditions were altered to 60 h at 45 °C to prevent blackening of the solution as a result of partial metal reduction to platinum(0), observed at higher reaction temperatures in previous experiments. The conjugate was isolated in 83% yield as a light-tan, water-soluble solid; η_{inh}, 10 ml g^{-1}. Analysis: Found: Pt, 8.9. Calcd for $(C_{91}H_{174}Cl_2N_{30}O_{23}Pt)_n$ (**3**) $(2322.5)_n$: Pt, 8.4%.

Conjugate 4

The carrier component of this conjugate, poly-α,β-D,L-[*N*-(3-(morpholin-4-yl)propylaspartamide (90)-*co*-*N*-(9-aza-3,6-dioxanonyl)aspartamide (10)], was synthesized as in the preceding experiment, except that 4-(3-aminopropyl)morpholine was used in place of the dimethylaminopropylamine. The yield was 64%; η_{inh}, 18 ml g^{-1}. Platination as described for **3** gave water-soluble conjugate **4** as a light-tan solid in 51% yield; η_{inh}, 13 ml g^{-1}. Analysis: Found: Pt, 7.7. Calcd for $(C_{109}H_{192}Cl_2N_{30}O_{32}Pt)_n$ (**4**) $(2700.8)_n$: Pt, 7.2%.

Conjugate 5

The polyamidoamine carrier component of **5** was prepared by an procedure described earlier[13] with minor modifications. Methylenebisacrylamide (1.542 g; 10 mmol) was dissolved in 40 ml of hot H_2O, and the solution was cooled to 0–5 °C, whereupon a portion of the solute crystallized out. To the stirred suspension was rapidly added Jeffamine ED 2001 (10 g; 5 mmol), and ethylene-

diamine (300 mg; 5 mmol), while nitrogen was introduced to saturation. The mixture was stirred in the stoppered flask for 18 h at 0 °C, 4 h at room temperature, and another 48 h at 65 °C. Ethanolamine (30 mg) was added and stirring continued for a further 4 h at that temperature in order to saturate any vinyl end-groups. Upon dilution with 25 ml of H_2O, the routinely filtered solution was dialyzed for 24 h in Spectra/Por 4 tubing against several batches of H_2O. The retentate was freeze-dried, and the crude product (5.9 g) was thoroughly washed with hot pentane for extraction of any admixed Jeffamine left unreacted. It was then redissolved in 30 ml of H_2O and redialyzed for two days in Spectra/Por 6 tubing. Freeze-drying of the retentate as before, followed by IR post-drying, gave 4.10 g (34.7%) of an off-white waxy solid completely soluble in H_2O. ^{1}H NMR (ppm): 4.6, 4.1H (4H; N—CH_2—N); 3.9–3.3, 171 H (171 H; CH—O, CH_2—O); 3.1–2.3, 19 H (22H; remaining CH, CH_2); 1.2–1.0, 9H (9H; CH_3).

A 473-mg portion (0.2 mmol) of the carrier so obtained was dissolved in 5 ml of H_2O. After the addition of K_2PtCl_4, (104 mg; 0.25 mmol), and saturation with nitrogen, the solution was stirred for 15 h at room temperature and for another 7 h at 65 °C in the dark. During the heating period, the pH was allowed gradually to decrease to 7; for the last 0.5 h, it was further lowered to 4–5 (HCl). The solution was dialyzed in Spectra/Por 4 tubing for 16 h against aqueous 0.15 M KCl and for another 40 h against H_2O acidified to pH 5 (HCl). Freeze-drying of the retentate left 390 mg (74.1%) of water-soluble, light-yellow solid; η_{inh}, 13 ml g^{-1}. Analysis: Found: Pt, 7.7. Calcd: Pt, 7.4%.

Conjugates 6 and 7

These were obtained previously as protonated species,[7] when they were designated **1** and **2**, respectively, each one containing 3 equiv of HCl per recurring unit (not shown in structures **6** and **7**). The polymers were redissolved in H_2O, and the filtered solutions were freeze-dried to afford the compounds as light-tan, water-soluble solids containing, respectively, 18.3 and 16.4% Pt.

Embedding

As a precautionary measure, the polyaspartamide-type conjugates **1–4, 6** and **7** were redissolved in H_2O together with three times their weight of NaCl, and the solutions were freeze-dried, affording each conjugate as a matrix-embedded solid. This embedding step served to prevent possible solid-state interaction during lengthy periods of overseas mailing from one laboratory (Johannesburg) to the other (Rehovot). Before the toxicological work, the samples were redissolved in H_2O and redialyzed for 18 h against H_2O for salt removal; they were recollected upon freeze-drying.

Toxicological procedures

The animals used were CD-1 male mice, 3–4 months old, with weights in the range of 18–25 g. Conjugates **1–7** were dissolved in phosphate-buffered saline (PBS) prior to injection; polymer concentrations ranged from 11 to 86 mg ml^{-1}. Mice were individually weighed, and conjugate solutions in volumes of 0.3–0.5 ml with increasing concentrations were injected intravenously into the tail vein. The animals were closely observed during the first 3 h post-injection, and daily observations were continued over a period of three weeks for changes in body weight and survival. Death of mice or decrease in body weight usually occurred during the first few days following administration. In a few cases, death was instantaneous, i.e. within minutes, presumably for reasons unrelated to drug toxicity, and these results were ignored. Animals surviving day 20 of the test and found to be indistinguishable from untreated mice were considered long-term survivors. Pertinent conditions and findings are summarized in Table 1. In this limited project the number of animals per conjugate was restricted to eight or less, although in repeated experiments of two or three mice per group.

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