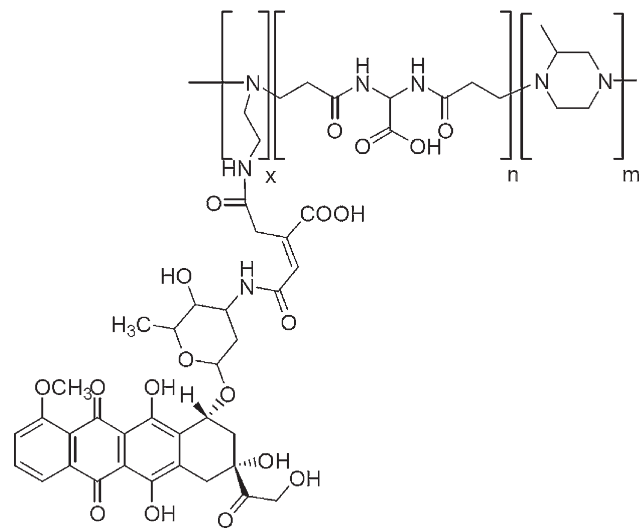
Poly(amidoamine) Conjugates Containing Doxorubicin Bound via an Acid-Sensitive Linker

Nathalie Lavignac,* Johanna L. Nicholls, Paolo Ferruti, Ruth Duncan

Poly(amidoamine)s with amino pendant groups were prepared by hydrogen-transfer polyaddition of primary and secondary amines to bis-acrylamines. Dansyl cadaverine (DC) doxorubicin (Dox) were bound to the polymers via a *cis*-aconityl spacer to give conjugates containing 3 μg of DC per mg of polymer and 28 to 35 μg of Dox per mg of polymer. Release of DC and Dox at physiological and acidic pH varied from 0 to 35% over 48 h and was pH dependent. Although the ISA1Dox conjugate ($IC_{50} = 6$ μg $Dox \cdot mL^{-1}$) presented similar toxicity as the parent polymer without Dox, ISA23Dox showed increased toxicity ($IC_{50} = 10$ μg $Dox \cdot mL^{-1}$). These results suggest that ISA23Dox is able to release biologically active Dox *in vitro* and that this conjugate might be suitable for further development.



Introduction

Following i.v. administration, low-molecular-weight drugs distribute into almost all tissues and intracellular compartments due to passive diffusion or active transport through cell membranes.[1] For anticancer agents such as anthracyclines this results in non-specific toxicity. When combined to polymeric carriers, the pharmacokinetics of the drug is modified at the body and cellular level and passive targeting to tumour site is obtained via the enhanced permeation and retention (EPR) effect.[2–4] Several macromolecular systems have been described to deliver doxorubicin (Dox)[5,6] including *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers[7,8] and poly (ethylene glycol) (PEG) conjugates.[9,10] Here, we described new polymer-Dox conjugates based on poly(amidoamine)s (PAAs).[11–12] PAAs are water-soluble polymers and are >100-fold less toxic [13,14] than other polycationic vectors.[15–17] Poly(amidoamine)s also present the advantage to degrade to oligomeric products in aqueous media within days or weeks, depending on their structures.[18,19] Owing to their capacity to undergo a conformational change from a coiled structure at pH = 7.4 to a more extended one when exposed to acidic pH,[20–22] most recent effort have been directed to develop stimuli-responsive constructs for biomacromolecules intracellular delivery.[23–26] However,

N. Lavignac, J. L. Nicholls, R. Duncan
Centre for Polymer Therapeutics, Welsh School of Pharmacy, Redwood Building, Cardiff University, King Edward VII Avenue, Cardiff, CF10 3XF, UK
N. Lavignac
School of Pharmacy, University of Kent, Central Avenue, Chatham Maritime, ME4 4TB, UK
Fax: +44 1634 883 927; E-mail: n.lavignac@kent.ac.uk
P. Ferruti
Dipartimento di Chimica Organica e Industriale and Centro Interdisciplinare Materiali Innovativi Nanostrutturati, Università di Milano, Via Venezian 21, 20133 Milano, Italy

DOI: 10.1002/mabi.200800163

these polymers have also the potential to be used as drug carrier. Poly(amidoamine)s with hydroxy pendant groups have been used to develop PAA/mitomycin (MMC) adducts[12,27] whereas PAAs containing β-cyclodextrin were used to deliver platinate (Pt) [28] and acyclovir.[29] Poly(amidoamine)/MMC conjugates were found to be less toxic than free MMC. When given by i.p. route, they were equi-active compare to MMC resulting in long-term survival of DBA2 mice bearing L1210 tumour cells and treated with the conjugate.[12] Similarly PAA/Pt were equi-active compare to cisplatin in an i.p. L1210 leukaemia model [28] and *in vitro*, PAA/acyclovir complexes exhibited a higher antiviral activity against herpes simplex viruses compare to the free drug.[29]

As Dox is inactive in its conjugated form,[8] tumour cell-specific released of the drug and subsequent biological activity must be achieved by the choice of a suitable degradable linker between the polymer and the drug. By taking advantage of the pH-gradient between the extra-cellular matrice and the endosome it could be possible to use a pH-sensitive spacer that would be stable at physiological pH whereas it would degrade in the acidic vesicles allowing the release of the drug in the cytosol.[30,31] Poly(amidoamine)-drug conjugates (Dox or dansyl cadaverin) were synthesised using a *cis*-aconityl linker. Stability of the conjugates was evaluated in solution at pH = 7.4 and 5. An *in vitro* cytotoxicity of Dox-based polymers was assessed using mouse melanoma B16F10 cells.

Experimental Part

Materials

Cis-aconitic anhydride and dansyl cadaverine (DC) were purchased from Fluka (Buchs, Germany). *N*-hydroxy-sulfosuccinamide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Pierce (Cramlington, UK). Triethylamine (TEA), sodium hydroxide (NaOH), Sephadex G25, gelonin, 5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) and Triton X-100 were all from Sigma (Dorset, UK). Dichloromethane, methanol and dialysing membrane were from Fisher (Loughborough, UK). The PD10 columns were from Pharmacia and PBS was supplied from Oxoid Ltd. (Basingstoke, UK). RPMI 1640 medium (25×10^{-3} M HEPES) supplemented with L-glutamine, foetal bovine serum (FBS) were purchased from Gibco-BRL (Paisley, UK). The B16F10 mouse melanoma cells were from ATCC (CRL-6475).

Synthesis of PAAs Conjugates

Synthesis of PAAs with Amino-Pendant Group

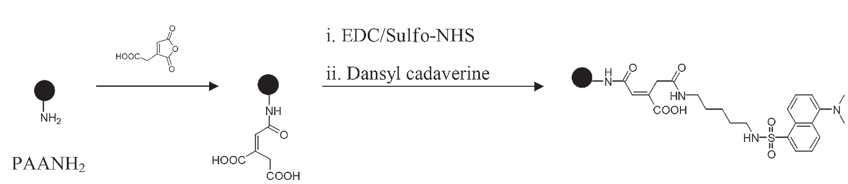
The PAAs with amino-pendant group ($ISA1NH_2$ and $ISA23NH_2$) were synthesised as described elsewhere.[32] The content of side chains terminated amino groups was determined using a ninhydrin assay.[33] Samples and 3-amino-1-propanol standards were prepared in water and 200 μL aliquots were added to Eppendorfs. After addition of 200 μL of ninhydrin reagent, solutions were incubated at 100 °C for 10 min. 300 μL of 50 vol.-% ethanol was then added after cooling on ice for 2 min. Absorbance was measured at 570 nm within 10 min. Unmodified PAAs, used as control, did not give false positive results.

Synthesis of ISA1-Dansyl Cadaverine

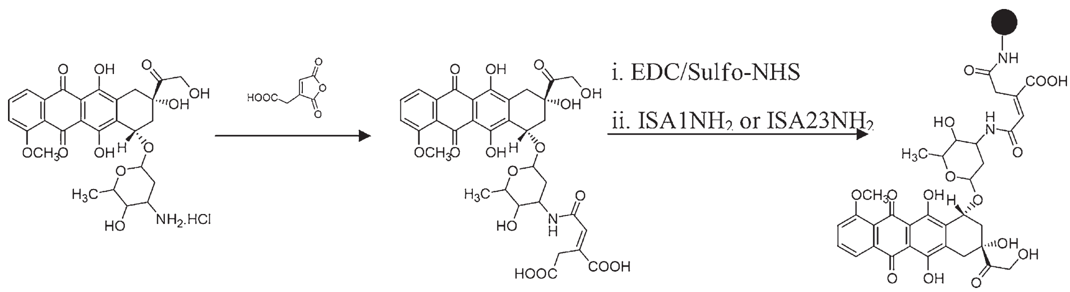
ISA1-dansyl cadaverine conjugate was synthesised as described in Scheme 1. Briefly, $ISA1NH_2$ (50 mg) was dissolved in 5 mL of PBS (0.1 M, pH = 8). The pH was adjusted back to 8 using 1 M NaOH. 50 mg of *cis*-aconitic anhydride (320 μmol) were added slowly whilst continually checking the pH and maintaining it between 8 and 8.5 using 1 M NaOH. The solution was left to react for 1 h at room temperature. The product was purified by gel permeation chromatography (GPC) on a Sephadex G25 column using PBS as eluent (0.1 M, pH = 7.4). Fractions containing the polymer were detected at 280 nm, pooled together and dialysed overnight against water (2 000 Da MW cut-off membrane). The final product was lyophilised. Conjugation of DC to the polymer derivative was carried out using a modified protocol adapted from Al-Shamkhani and Duncan.[34] ISA1-*cis*-aconityl (30 mg) was dissolved in 1.9 mL carbonate buffer (0.1 M, pH = 9). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.78 mg, 4.1 μmol) was added. The solution was stirred for 2 min. at room temperature and sulfo-NHS (0.82 mg, 3.8 μmol) was added. The pH was adjusted to pH = 9 using NaOH (1.0 M). The solution was stirred at room temperature for 1 h after which DC (1.2 mg, 3.6 μmol) was added and the mixture stirred in the dark for a further 1.5 h. The conjugate was purified by GPC. Fractions containing the conjugate were detected by fluorescence at 520 nm (excitation wavelength at 300 nm). The fractions were pooled and lyophilised. The recovered product was desalted using a PD10 column and lyophilised.

Synthesis of PAA-Dox

Poly(amidoamine)-Dox was synthesised as described in Scheme 2. Doxorubicin-*cis*-aconityl was prepared using the method of Shen and Ryser with some modifications.[35] Doxorubicin hydrochloride (50 mg, 86.2 μmol) was dissolved in 15 mL of ice-cold carbonate buffer (0.1 M, pH = 9). *Cis*-aconitic anhydride (50 mg, 0.32 mmol) was added slowly at 0 °C while maintaining a pH of 8.5 by addition of ice-cold NaOH (0.5 M). The reaction mixture was stirred at 0 °C for 20 min. and then at room temperature (20 min.). The reaction was cooled on ice and acidified with ice-cold HCl (1 M) till precipitation. The precipitate formed was isolated by centrifugation (10 min at 4 000 g and 4 °C), dissolved in doubled distilled water and recovered by lyophilisation. Conjugation of Dox-*cis*-aconityl to PAA was carried out as previously described.[36] Doxorubicin-*cis*-aconityl (7 mg, 10 μmol) was dissolved in 12 mL of



Scheme 1. Synthesis of ISA1DC conjugate.



Scheme 2. Synthesis of ISA23Dox and ISA1Dox conjugates.

PBS (0.1 M, pH = 7.4). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (20 mg, 0.1 mmol) was added. The solution was stirred for 2 min at room temperature and sulfo-NHS (25 mg, 0.12 mmol) was added. The pH was adjusted to pH = 9 using NaOH (1.0 M). The solution was stirred at room temperature for 30 min. after which ISA1-NH_2 or ISA23-NH_2 (100 mg) was added. pH was adjusted to neutral with NaOH (1 M) and the mixture stirred for 20 h in the dark. The conjugate was purified by GPC. Fractions containing the polymer were pooled and lyophilised. The recovered product was desalted using a PD10 column and relyophilised.

Characterisation of PAAs Conjugates

Determination of Doxorubicin or Dansyl Cadaverine Content

Dansyl cadaverine and Dox content in the conjugates were estimated by UV spectrophotometry at 485 (Dox) and 335 nm (DC) using free DC or free Dox as standards.[37]

Determination of Free Doxorubicin or Free Dansyl Cadaverine

The amount of free drug in the conjugates was determined by reverse-phase HPLC after extraction in organic solvent.[38] Briefly, 100 μL of ammonium formate (1 M, pH = 8.5) was added to the conjugates (0–5 mg) dissolved in water (900 μL). For Dox containing samples, daunomycin (500 ng) was used as internal standard. After addition of 5 mL of chloroform, the samples were mixed and centrifuged for 10 min. at 13 000 rpm. The aqueous phase was discarded, the solvent evaporated under nitrogen and the residues re-dissolved in methanol (100 μL). Recovered-free DC and Dox were quantified by HPLC using a Waters Spherisorb 5 μm ODS2 column (150×3.9 mm^2). For DC, the mobile phase (H_2O/methanol/TEA, 70:28:2 by volume) was delivered at a flow rate of 1 mL · min^{-1} with a Jasco PU-980 Intelligent HPLC pump. DC fluorescence was detected at 508 nm with an excitation wavelength at 336 nm using a GBC LC1255 Fluoro detector. For Dox, the mobile phase was propanol/H_2O, 29:71 vol.-%, the pH was adjusted to 3.5 with orthophosphoric acid. Dox fluorescence was detected at 560 nm with an excitation wavelength at 480 nm.

Release Study of DC or Dox from Conjugates at Different pH

Stability of the *cis*-aconytil linker was evaluated in buffer solutions at several pH. ISA1DC (7 mg · mL^{-1}, 21.77 μg of DC per mL), ISA1Dox (4 mg · mL^{-1}, 357.5 μg of Dox per mL) and ISA1Dox (4 mg · mL^{-1}, 281.7 μg of Dox per mL) were dissolved in citrate/phosphate buffers 0.1 M at pH = 5 and 7.4. The solutions were incubated in a water bath at 37 °C. Aliquots (100 μL) were removed at different time points, immediately frozen with liquid nitrogen and stored into a freezer (−80 °C) until further analysis. Free DC and Dox were extracted and quantified by HPLC as described before.

Evaluation of *in vitro* Cytotoxicity of Doxorubicin Conjugates

The cytotoxicity of the PAADox conjugates was assessed using a murine melanoma B16F10 model. Cells were cultured in RPMI-1640 supplemented with 5×10^{-3} M L-glutamine 10 vol.-% heat-inactivated FBS and maintained at 37 °C in a humid incubator with a 5% CO_2 atmosphere. No antibiotics were added. Polymer cytotoxicity was assessed during the log phase of cell growth using an MTT assay as described previously.[25] Cells were added to 96-well microtitre plates at a density of 1×10^4 cells · $well^{-1}$ 24 h prior to the assay. Polymer solutions (0.2 μm filtered) were made in complete RPMI-1640 medium to give a concentration range of 0 to 4 mg of polymer per mL. At the start of the experiment the culture medium was removed and the desired polymer solution was added (100 μL). After 67 h, MTT (20 μL; 5 mg · mL^{-1} in PBS sterile filtered) was added to each well and the plates re-incubated for a further 5 h. The formazan crystals were dissolved in DMSO and concentration read at 550 nm using a microtitre plate reader. The results were expressed as per cent viability relative to a control containing no polymer (i.e. cells grown in media alone were used as a reference for 100% viability).

Results and Discussion

The concept of polymer drug carrier is not new; Ringsdorf proposed it 30 years ago.[39] He described his model as a water-soluble macromolecular prodrugs consisting of an inert carrier to which the drug is attached directly or via a degradable spacer. A targeting moiety can be additionally added for cell-specific delivery via receptor-mediated endocytosis. Over the year, several systems using passive or enzymatic hydrolysis as well as pH controlled release

DOI: 10.1002/mabi.200800163

and reduction sensitive spacers were developed.[31] Proteolytically degradable bonds are generally used for lysosomotropic drug delivery and peptidyl linkers, such as Gly-Phe-Leu-Gly which is degraded by lysosomal enzymes such as cathepsin B, are amongst the most widely used spacers.[30] However, for cells with limited content of lysosomes, this might preclude the therapeutic effect of such macromolecular prodrugs.[40] Furthermore, recent results suggest that the *in vivo* enzymatic degradation of this type of linker relying on cathepsin B might be gender dependant.[41] *N*-(2-hydroxypropyl)methacrylamide-Dox conjugates prepared with pH sensitive spacers, hydrazone bond[42] and *cis*-aconityl[43] were found to be more cytotoxic than PK1 (an HPMA-dox conjugate using a pedtidyl linker) probably due to a higher release rate of the drug. Polymer conjugates using a pH sensitive linker could therefore present some advantages. In this paper, we report the synthesis and preliminary evaluation of PAAs-based conjugates containing either DC or Dox linked to the polymer backbone via a *cis*-aconityl linker. DC was initially chosen as a model molecule. It was later replaced by Dox due to its potential therapeutic application in chemotherapy.

Poly(amidoamine)s containing amino groups in their side chains (Figure 1) were synthesised by hydrogen transfer polyaddition of aliphatic amines to bisacrylamides.[32] The molecular weight ($\overline{M}_w$) of ISA23NH$_2$ and ISA1NH$_2$ were 22.7 and 16.7 kDa, respectively; their polydispersity indices were 1.9 and 1.7 (Table 1). The amine function was introduced using 1-triphenylmethylamino-2-aminoethane (TPHMAE) as comonomer (5 mol-% theoretically). The content of incorporated TPHMAE was estimated using a ninhydrin assay as described in the methods. ISA23NH$_2$ and ISA1NH$_2$ contained 4 and 4.5 mol-% of amino groups, respectively. Synthesis of the conjugates was carried out using sequential procedures as described in Scheme 1 and 2. DC-based polymer was synthesised via a polymer precursor obtained by the reaction of *cis*-aconitic anhydride with the amine groups of ISA1NH$_2$ (Scheme 1). Dansyl cadaverine was conjugated to

Table 1. Physicochemical characterisation of the PAAs.

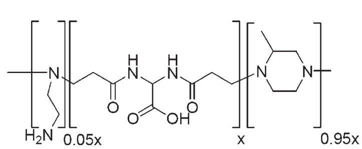
Polymers	$\overline{M}_w$ [a)]	PDI[a)]	NH$_2$ content[b)] mol-%	Drug content[c)] wt.-%
ISA1	39 200	1.72	–	–
ISA23	38 800	1.75	–	–
ISA1NH$_2$	16, 700	1.70	4.5	–
ISA23NH$_2$	22, 700	1.98	4	–
ISA1DC	16, 100	1.84	–	0.3
ISA1Dox	17, 800	1.87	–	3.6
ISA23Dox	23, 500	2.31	–	2.8

[a)]$\overline{M}_w$ and PDI (polydispersity index) were determined by GPC using poly(*N*-vinylpyrrolidone) standards; [b)]Content of pendant amino groups; [c)]DC or Dox.

the precursor after activation of the carboxy groups with EDC and sulfo-NHS. Unreacted DC was removed by Sephadex G25 column chromatography and the resulting conjugate was desalted using a PD10 column (Figure 2). Dox-based polymers were prepared using a similar procedure (Scheme 2), respective polymers were reacted with *N*-*cis*-aconityl Dox, prepared by acylation of the amino group of Dox-HCl.[35] Drug loading determined spectrophotometrically was 3.11 μg of DC per mg of polymer for ISA1DC conjugate, 35.72 μg of Dox per mg of polymer for ISA1-*cis*-Dox conjugate and 28.17 μg of Dox per mg of polymer for ISA23Dox conjugate. No detectable drug (less than 0.1 wt.-% free drug relative to total DC or Dox) was found in conjugates after purification.

Doxorubicin is inactive in its conjugated form and to exert its biological activity it has to be released from the polymer backbone.[8] However, after i.v. injection, the conjugate must be stable while in the blood stream and to avoid unspecific cytotoxicity release must only occur in the acidic compartments of the tumour cells. Stability of the pH sensitive linker was though examined at 37 °C in citrate/phosphate buffers at pH values of 5 and 7.4 to mimic endosomes and extracellular pHs (Figure 3 and 4). The amount of drug released from the PAAs conjugates was quantified by HPLC. As expected the release profiles depended on the pH. For all conjugates the amount of drug released was lower at pH = 7.4 with less than 3% of drug released within 42 h. At acidic pH, hydrolysis of the *cis*-aconityl spacer occurred slowly in all cases, although the release rates were

ISA23NH$_2$



ISA1NH$_2$

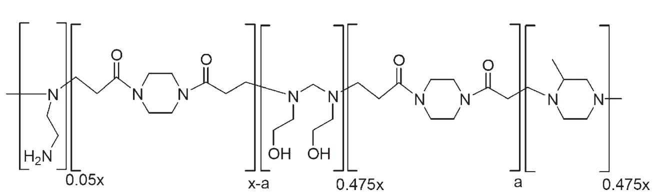


Figure 1. Structure of the PAAs.

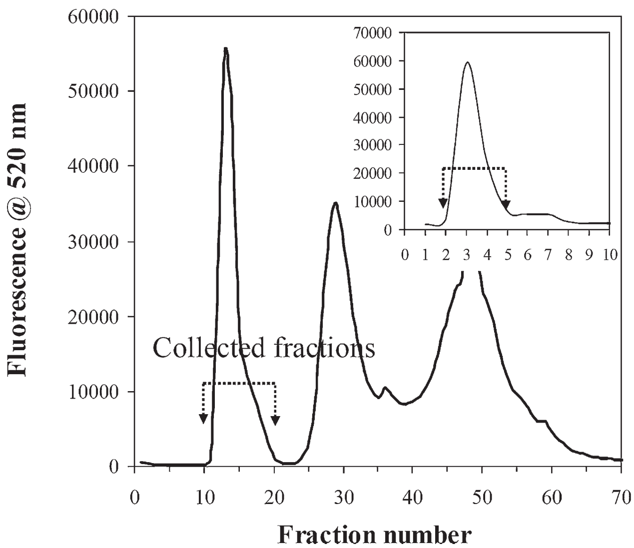


Figure 2. Purification of the conjugate ISA1DC by GPC on a Sephadex G25 column using PBS as eluent (0.1 M, pH = 7.4). Inset: elution profile after desalting on the PD10 column.

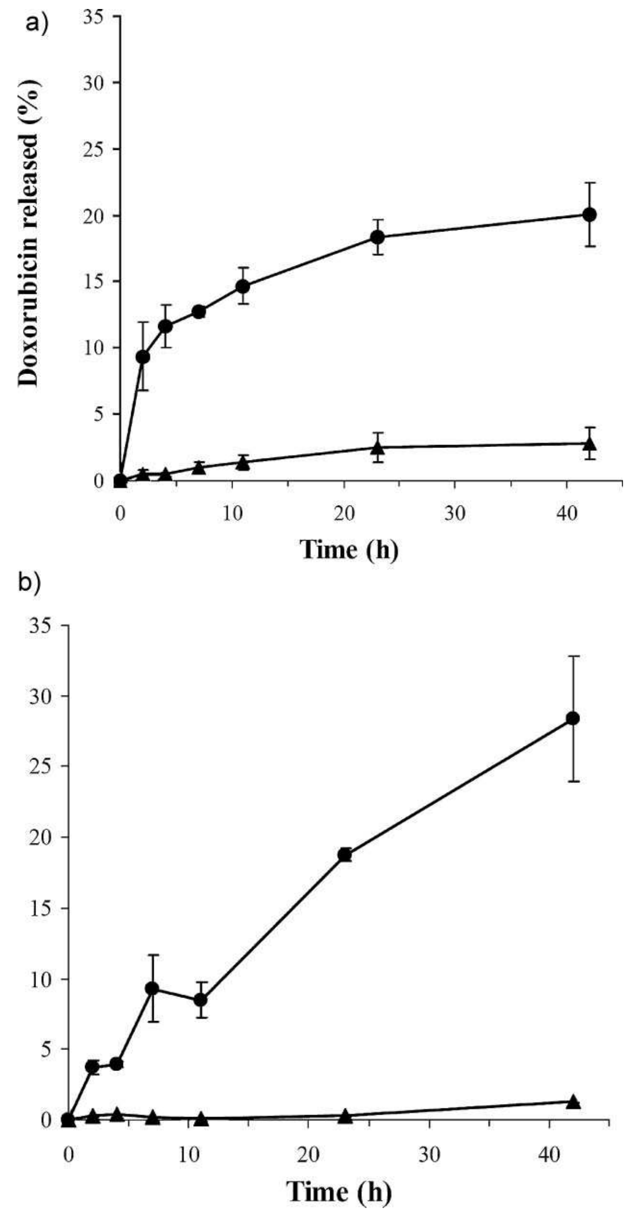


Figure 4. Release study of Dox from (a) ISA1Dox and (b) ISA23Dox. Stability of the *cis*-aconityl linker at 37 °C, was investigated at pH = 5 (●) and pH = 7.4 (▲) (Data represents mean ± SEM; $n = 6$).

different for each conjugate. For ISA1DC polymer (Figure 3) the release started after a time lag of 6 h whereas for Dox-based conjugates degradation of the *cis*-aconityl linker had already occurred after 2 h of incubation. For ISA23Dox (Figure 4b) the release increased regularly over the period of incubation with 30% of Dox released after 42 h. For ISA1, the release profile was different (Figure 4a). Nearly 10% of Dox was released within 2 h compare to less than 4% for ISA23. After 24 h of incubation, the release of Dox was quite steady and the concentration of Dox in solution reached a plateau at approximately 20%.

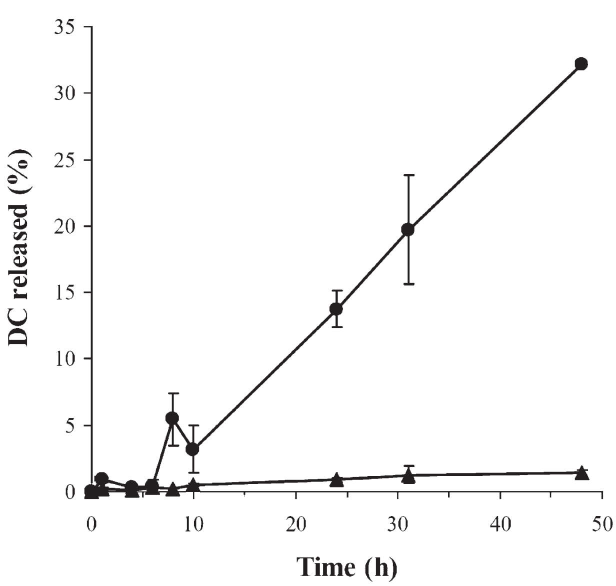


Figure 3. Release study of DC from ISA1DC. Stability of the cis aconityl linker at 37 °C, was studied at pH = 5 (●) and pH = 7.4 (▲). (Data represents mean ± SEM; $n = 6$).

Shen and Ryser[35] were the first to describe the synthesis of poly(D-lysine)-daunomycin (DNM) conjugates using a *cis*-aconytil spacer. Similarly, they found that the linker was more stable at pH = 6 than at pH = 4 with a half-life of more than 96 h in the first case and less than 3 h in the second. Further *in vitro* studies, demonstrated the release of DNM in the endosome compartment. To evaluate the release of Dox *in vitro*, the cytotoxicity of the PAA-*cis*-Dox conjugates synthesised was assessed

DOI: 10.1002/mabi.200800163

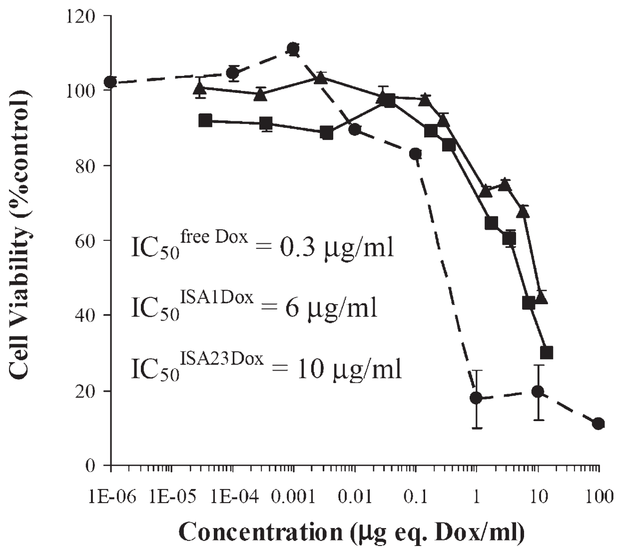


Figure 5. Cytotoxicity of PAADox relative to free Dox. Viability is expressed as per cent of the growth of control B16F10 mouse melanoma cells incubated in medium alone. IC_{50}: Dox concentration at which 50% of the cells are dead. ISA23Dox (▲), ISA1Dox (■) and Free Dox (●) (data represents mean ± SEM; $n = 6$).

using a murine melanoma B16F10 model and an MTT assay. Free Dox and parent polymers ISA1, $ISA1NH_2$, ISA23 and $ISA23NH_2$ were used as control. Cytotoxicity of both conjugates ($IC_{50} = 6$ μg $Dox \cdot mL^{-1}$ for ISA1Dox and $IC_{50} = 10$ μg $Dox \cdot mL^{-1}$ for ISA23Dox) was lower compare to free Dox ($IC_{50} = 0.3$ μg $Dox \cdot mL^{-1}$) (Figure 5). This has been reported before for other polymer-Dox conjugates.[10] It is due to low rate of endocytic uptake and endosomotropic/lysosomotropic activation as the limiting rate for the conjugate compare to the free drug that can diffuse through cell's membrane. For conjugate ISA1Dox the toxicity was similar to that of the parent polymers ($IC_{50} = 1.5$ mg $polymer \cdot mL^{-1}$) (Figure 6a). Therefore the toxicity could not be correlated with the release of Dox. For conjugate ISA23Dox, cell death was dose dependent (Figure 6b) and cell death increased compare to the parent polymers that were not toxic (ISA23; $ISA23NH_2$) under the same conditions. These results confirmed the release of the drug from the polymer backbone (i.e. ISA23Dox) after degradation of the *cis*-aconityl linker in the endosomal/lysosomal compartments.

Our results suggest that the mechanism of Dox release from the polymer backbone (ISA23 or ISA1) is different (Figure 4) and that the conjugates present different biological activity (Figure 6). It has been showed that when daunomycin is acylated with *cis*-aconitic anhydride two isomers are obtained (*cis*-DNM and *trans*-DNM).[44] Recently, Kakinoki et al.[45] reported similar results for Dox. They conjugated Dox to poly(vinyl alcohol) (PVA) via a *cis*-aconityl linker. They found that in solution, the configuration of the intermediate isomers (*cis*-Dox and

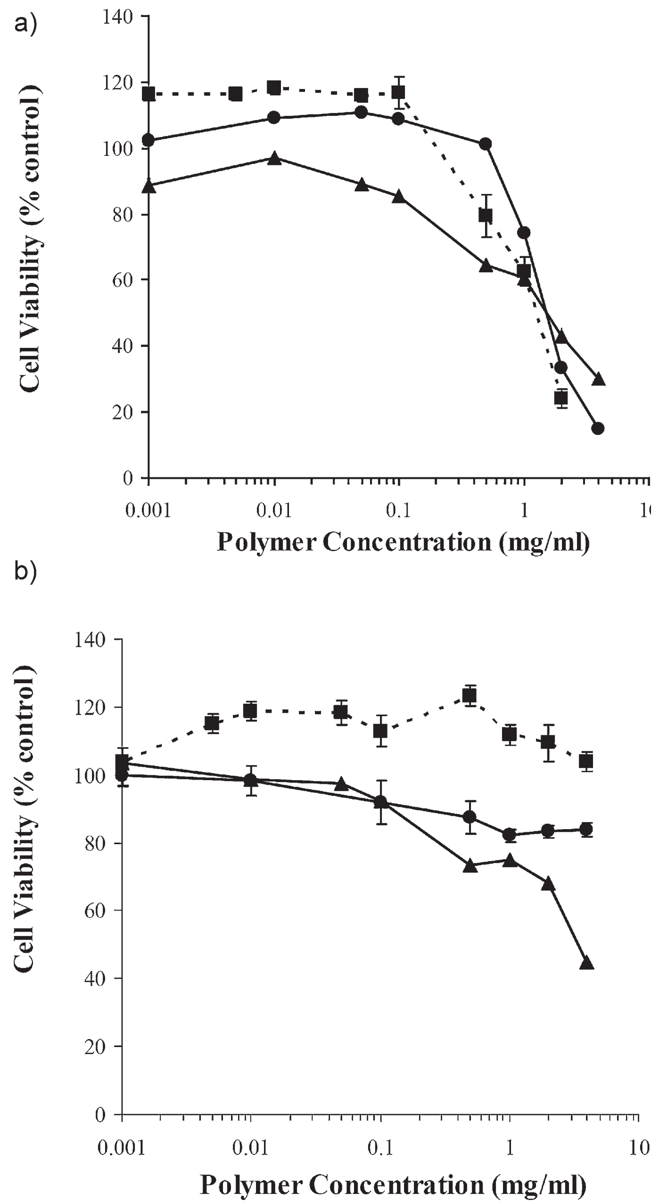


Figure 6. Cytotoxicity of PAAss toward B16F10 mouse melanoma. Viability is expressed as per cent of the growth of control cells incubated in medium alone. Panel (a) ISA1 (■), $ISA1NH_2$ (●) and ISA1Dox (▲). Panel (b) ISA23 (■), $ISA23NH_2$ (●) and ISA23Dox (▲) (data represents mean ± SEM; $n = 6$).

trans-Dox) had an influence on the kinetics release profile of Dox from the polymer. At pH = 5 the half-life for the release of Dox was 3 h for PVA-*cis*-Dox whereas it was 14 h for PVA-*trans*-Dox. They concluded that the *cis* conformation catalysed the hydrolysis of the amide bond. They also demonstrated that the biological activity of the polymer conjugate (PVADox) depended on the configuration of the aconityl-Dox isomer. Under similar conditions the *cis*-Dox conjugate displayed higher toxicity. In the present study, we did not isolate the isomers (*cis*-Dox and *trans*-Dox).

The proportion of *cis*-Dox and *trans*-Dox might therefore be different between the two conjugates, which could potentially explain the difference between their release profile and activity.

ISA1 and ISA23 are poly(amidoamine)s with different chemical structure. This could also alter the biological activity of the Dox conjugates. Several studies have shown that the structure of polymers can have an effect on their cellular uptake and that polymers with different architecture may have different intracellular trafficking.[46,47] Our conjugates are stimuli responsive. The release of Dox is pH dependent and requires substantial access to the acidic compartment of the cells. It has already been suggested in a previous study that the intracellular fate of ISA1 and ISA23 polymers may be different.[24] Such variation in trafficking may affect the kinetics of Dox release *in vitro* and could also explain the difference of activity between ISA1Dox and ISA23Dox. Two HPMA conjugates (PK1 and PK2) with similar chemical structure displayed different maximum-tolerated dose (MTD) in clinical trials. A recent study using small angle neutron scattering (SANS) suggested that this difference might be explained by different conjugates conformation.[48] Such experiments have already shown the pH-dependent changes in conformation of poly(amidoamine)s,[20–22] experiments with the present conjugates could lead to further insight into the mechanism of Dox release.

Conclusion

Poly(amidoamine)s/Dox conjugates (ISA1Dox and ISA23-Dox) were synthesised from PAAs with amino pendant groups and Dox acylated with *cis*-aconitic anhydride. Although both conjugates demonstrated pH-dependent stability in buffer solutions, only ISA23Dox showed the ability to release biologically active Dox in the endosomal compartment of B16F10 cells. Influence of the Dox isomer's configuration (*cis*-Dox or *trans*-Dox) is currently under investigation. Macromolecular systems are quite often eliminated from the blood circulation by the reticuloendothelial system. However, ISA23 is known to possess enhanced blood circulation and *in vivo* experiments using animal models have shown it does accumulate in tumours. It would be though also interesting to undertake pharmacokinetic studies to see if the ISA23Dox conjugate accumulates in tumours and to evaluate the fate of the remaining drug bearing polymer.

Acknowledgements: We thank *BBSRC* for supporting this work.

Received: June 17, 2008; Revised: October 1, 2008; Accepted: October 7, 2008; DOI: 10.1002/mabi.200800163

Keywords: drug delivery systems; nanotechnology; polyelectrolyte; stimuli-sensitive polymers; water-soluble polymers

[1] R. Duncan, "Targeting and Intracellular Delivery of Drugs", in: *Encyclopedia of Molecular Cell Biology and Molecular Medicine*, R. A. Meyers, Ed., WILEY-VCH Verlag, GmbH & Co. KGaA, Weinheim 2005, p. 163.
[2] A. Nori, J. Kopecek, *Adv. Drug Delivery Rev.* **2005**, *57*, 609.
[3] R. Duncan, *Nature Rev. Drug Discov.* **2003**, *2*, 347.
[4] Y. Matsumara, H. Maeda, *Cancer Res.* **1986**, *6*, 6387.
[5] C. Rousselle, M. Smirnova, P. Clair, J. M. Lefauconnier, A. Chavanieu, B. Calas, J. M. Scherrmann, J. Temsamani, *J. Pharmacol. Exp. Ther.* **2001**, *296*, 124.
[6] L. D. Mayer, G. Dougherty, T. O. Harasym, M. B. Bally, *J. Pharmacol. Exp. Ther.* **1997**, *280*, 1406.
[7] P. A. Vasey, S. B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A. H. Thomson, L. S. Murray, T. E. Hilditch, T. Murray, S. Burtles, R. Fraier, E. Frigero, J. Cassidy, *Clin. Cancer Res.* **1999**, *5*, 83.
[8] A. Malugin, P. Kopeckova, J. Kopecek, *J. Controlled Release* **2007**, *124*, 6.
[9] M. Pechar, A. Braunova, K. Ulbrich, M. Jelinkova, B. Rihova, *J. Bioact. Compat. Polym.* **2005**, *20*, 319.
[10] F. M. Veronese, O. Schiavon, G. Pasut, R. Mendichi, L. Andersson, A. Tsirk, J. Ford, G. F. Wu, S. Kneller, J. Davies, R. Duncan, *Bioconjugate Chem.* **2005**, *16*, 775.
[11] J. Franchini, P. Ferruti, *J. Bioact. Compat. PolYM.* **2004**, *19*, 221.
[12] P. Ferruti, M. Marchisio, R. Duncan, *Macromol. Rapid Commun.* **2002**, *23*, 332.
[13] E. Ranucci, G. Spagnoli, P. Ferruti, D. Sgouras, R. Duncan, *J. Biomater. Sci. Polym. Ed.* **1991**, *2*, 303.
[14] P. Ferruti, R. Duncan, S. C. W. Richardson, "*Targeting of Drugs 6: Strategies for Stealth Therapeutics Systems*", G. Gregoriadis, B. McCormack, Eds., Plenum Press, New York 1998, p. 207.
[15] G. Wu, C. Wu, *J. Biol. Chem.* **1987**, *262*, 4429.
[16] O. Boussif, F. Lezoualch, M. Zanta, M. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Nat. Acad. Sci. USA* **1995**, *92*, 7297.
[17] J. D. Eichman, A. U. Bielinska, J. F. Kukowska-Latallo, J. R. Baker, *Pharm. Sci. Technol. Today* **2000**, *3*, 232.
[18] P. Ferruti, E. Ranucci, F. Bignotti, L. Sartore, P. Bianciardi, M. A. Marchisio, *J. Biomater. Sci. Polym. Ed.* **1994**, *6*, 833.
[19] P. Ferruti, E. Ranucci, L. Sartore, F. Bignotti, M. A. Marchisio, P. Bianciardi, F. M. Veronese, *Biomaterials* **1994**, *15*, 1235.
[20] P. C. Griffiths, A. Paul, Z. Khayat, K. W. Wan, S. M. King, I. Grillo, R. Schweins, P. Ferruti, J. Franchini, R. Duncan, *Biomacromolecules* **2004**, *5*, 1422.
[21] Z. Khayat, P. C. Griffiths, I. Grillo, R. K. Heenan, S. M. King, R. Duncan, *Int. J. Pharm.* **2006**, *317*, 175.
[22] P. C. Griffiths, Z. Khayat, S. Tse, R. K. Heenan, S. M. King, R. Duncan, *Biomacromolecules* **2007**, *8*, 1004.
[23] S. C. W. Richardson, N. G. Pattrick, Y. K. S. Man, P. Ferruti, R. Duncan, *Biomacromolecules* **2001**, *2*, 1023.
[24] N. G. Pattrick, S. C. W. Richardson, M. Casolaro, P. Ferruti, R. Duncan, *J. Controlled Release* **2001**, *77*, 225.
[25] N. Lavignac, M. Lazenby, P. Foka, B. Malgesini, I. Verpilio, P. Ferruti, R. Duncan, *Macromol. Biosci.* **2004**, *10*, 922.
[26] N. Lavignac, M. Lazenby, J. Franchini, P. Ferruti, R. Duncan, *Int. J. Pharm.* **2005**, *300*, 102.
[27] E. Schacht, P. Ferruti, R. Duncan, *Chem. Abstr.* **1995**, *595*, 248301a, WO 9505,200.
[28] P. Ferruti, E. Ranucci, F. Trotta, E. Gianasi, E. G. Evagorou, M. Wasil, G. Wilson, R. Duncan, *Macromol. Chem. Phys.* **1999**, *200*, 1644.

[29] M. Bencini, E. Ranucci, P. Ferruti, F. Trotta, M. C. Donalisio, D. Lembo, R. Cavalli, *J. Controlled Release* **2008**, *126*, 17.
[30] S. Brocchini, R. Duncan, "Polymer Drug Conjugates: Drug Release from Pendent Linkers", in: *Encyclopedia of Controlled Release*, E. Mathiowitz, Ed., *Wiley*, New York 1999, p. 786.
[31] H. Soyez, E. Schacht, S. Vanderkerken, *Adv. Drug Del. Rev.* **1996**, *21*, 81.
[32] B. Malgesini, I. Verpilio, R. Duncan, P. Ferruti, *Macromol. BioSci.* **2003**, *3*, 59.
[33] M. M. Leane, R. Nankervis, A. Smith, L. Illum, *Int. J. Pharm.* **2004**, *271*, 241.
[34] A. Al-Shamkhani, R. Duncan, *Int. J. Pharm.* **1995**, *122*, 107.
[35] C. W. Shen, H. J. P. Ryser, *BBRC* **1981**, *102*, 1048.
[36] K. Ulbrich, T. Etrych, P. Chytil, M. Jelinkova, B. Rihova, *J. Controlled Release* **2003**, *87*, 33.
[37] F. Searle, S. Gac-Breton, R. Keane, S. Dimitrijevic, S. Brocchini, E. A. Sausville, R. Duncan, *Bioconjug. Chem.* **2001**, *12*, 711.
[38] R. Duncan, L. W. Seymour, K. B. O'Hare, P. A. Flanagan, S. Wedge, I. C. Hume, K. Ulbrich, J. Strohalm, V. Subr, F. Spreafico, M. Grandi, M. Ripamonti, M. Farao, A. Suarato, *J. Controlled Release* **1993**, *19*, 331.
[39] H. Ringsdorf, *J. Polym. Sci. Polym. Symp.* **1975**, *51*, 135.
[40] B. Rihova, T. Etrych, M. Pechar, M. Jelinkova, M. Stastny, O. Hovorka, M. Kovar, K. Ulbrich, *J. Controlled Release* **2001**, *74*, 225.
[41] S. Di Giovine, P. De Feudis, D. Torriani, G. Colella, M. Cassin, L. Piazzoni, U. Bastrup, M. Natangelo, G. Pezzoni, J. Singer, *Eur. J. Cancer Suppl.* **2006**, *4*, 191.
[42] M. Kovar, L. Kovar, V. Subr, T. Etrych, K. Ulbrich, T. Mrkvan, J. Loucka, B. Rihova, *J. Controlled Release* **2004**, *99*, 301.
[43] W. M. Choi, P. Kopeckova, T. Minko, J. Kopecek, *J. Bioact. Compat. Polym.* **1999**, *14*, 447.
[44] J. Remenyi, B. Balazs, S. Toth, A. Falus, G. Toth, F. Hudecz, *Biochem. Biophys. Res. Commun.* **2003**, *303*, 556.
[45] A. Kakinoki, Y. Kaneo, Y. Ikeda, T. Tanaka, K. Fujita, *Biol. Pharm. Bull.* **2008**, *31*, 103.
[46] P. Seib, A. T. Jones, R. Duncan, *J. Controlled Release* **2007**, *117*, 291.
[47] M. Xyloyannis, O. L. Padilla De Jesus, J. M. J. Frechet, R. Duncan, *Proc. Int. Symp. Controlled Release Bioact. Mater.* **2003**, *30*, 149.
[48] A. Paul, M. J. Vicent, R. Duncan, *Biomacromolecules* **2007**, *8*, 1573.