Glycobiology vol. 22 no. 10 pp. 1363–1373, 2012
doi:10.1093/glycob/cws091
Advance Access publication on May 30, 2012

Improved bioavailability of inhibitors of *Trypanosoma cruzi* *trans*-sialidase: PEGylation of lactose analogs with multiarm polyethyleneglycol

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Received on May 4, 2012; revised on May 24, 2012; accepted on May 26, 2012

The *trans*-sialidase of *Trypanosoma cruzi* (TcTS) catalyzes the transfer of sialic acid from host glycoconjugates to terminal β-galactopyranosides in the mucins of the parasite. During infection, the enzyme is actively shed by the parasite to the bloodstream inducing hematological alterations. Lactitol prevents cell apoptosis caused by the TcTS, although it is rapidly eliminated from the circulatory system. Linear polyethyleneglycol (PEG) conjugates of lactose analogs were prepared but their clearance from blood was still quite fast. With the aim of improving their circulating half-lives in vivo, we now synthesized covalent conjugates of eight-arm PEG. The star-shape of these conjugates allows an increase in the molecular weight together with the loading of the active sugar. Two approaches were used for PEGylation of disaccharide derivatives containing β-D-Gal*p* as the non-reducing unit. (1) Amide formation between benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside and a succinimide-activated PEG. (2) Conjugation of lactobionolactone with amino end-functionalized PEG. Two 8-arm PEG derivatives (20 and 40 kDa) were used for each sugar. Substitution of all arms was proved by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The bioavailability of the conjugates in mice plasma was considerably improved with respect to the 5 kDa linear PEG conjugates retaining their inhibitory properties.

Keywords: inhibitors / multiarm conjugates / PEGylation / *trans*-sialidase / *Trypanosoma cruzi*

Introduction

Glycans have been recognized as candidates for chemotherapy (Kawasaki et al. 2009). In this respect, *Trypanosoma cruzi,* the agent of American trypanosomiasis (Moncayo 2003) expresses a unique enzyme, the *trans*-sialidase (TcTS), involved in the transfer of sialic acid from host glycoconjugates to β-galactopyranosyl units in the mucins of the parasite (Schenkman et al. 1991; Frasch 2000; Giorgi and de Lederkremer 2011). The 3D structure of TcTS shows two (sub) sites in the active center: the sialic acid-binding site and the galactose-binding site (Buschiazzo et al. 2002; Amaya et al. 2003). Inhibitors of TcTS binding to the β-galactosyl acceptor site should be more specific, as other sialidases lack this interaction. Oligosaccharides of the mucins have been synthesized and their acceptor and inhibitory properties were studied (Agusti et al. 2007; Campo et al. 2007; de Lederkremer and Agusti 2009). Recently, systematically modified octyl-β-D-galactopyranosides, including disaccharides, were studied as substrates and inhibitors of TcTS (Harrison et al. 2011). Conjugates of sialic acid with galactose and other monosaccharides have been obtained by click chemistry (Campo et al. 2012). The triazole–sialic acid derivatives containing galactose showed high inhibitory values at 1.0 mM concentrations on TcTS-catalyzed hydrolysis of 4-methylumbelliferyl-*α*-D-*N*-acetylneuraminic acid. Lactose derivatives effectively inhibited the transfer of sialic acid to *N*-acetyllactosamine (Agusti et al. 2004). The best one tested, lactitol, also prevented the apoptosis caused by the TcTS (Mucci et al. 2006) despite it being rapidly eliminated from the circulatory system.

Synthetic oligovalent glycoconjugates have been prepared with the aim to increase the affinity of the carbohydrate to protein targets (Kitov et al. 2000; Chabre and Roy 2010). In this direction, a recent paper described the synthesis of 1,6-linked cyclic pseudo-galactooligosaccharides and their in vitro sialylation by recombinant TcTS (Campo et al. 2010). The synthesis of tetravalent glycoclusters with β-lactosyl residues and their trypanocidal activity has recently been described (Galante et al. 2011). Modification of biological molecules by covalent conjugation with polyethyleneglycol (PEG) has been used to improve the bioavailability of drugs (Veronese and Mero 2008). PEG is a linear polyether diol with many useful properties such as biocompatibility, solubility in aqueous and organic media, lack of toxicity and very low immunogenicity. Probably, the most important feature of

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PEG modification is that it greatly extends the half-life ($t_{1/2}$) of most proteins, and results in an increased presence in plasma. This can be attributed, in part, to the increase in molecular weight of the conjugate beyond the limit of renal filtration and to reduced proteolysis of the conjugate (Greenwald 2001). PEG has been coupled to antibodies or antibody fragments to prolong the circulating half-lives in vivo (Chapman 2002). Selective alkylation and acylation of amino groups in a somatostatin analog using two different PEG reagents have been described (Morpurgo et al. 2002). Also, low molecular weight drugs have been PEGylated in order to prolong the in vivo action (Marcus et al. 2008) or for targeting drug delivery (Dixit et al. 2006). A multifunctional PEG was prepared by a click reaction and coupled to dexamethasone for treatment of rheumatoid arthritis (Liu et al. 2010).

There are few reports on the PEGylation of carbohydrate molecules and these have been focused on polysaccharides or on carbohydrates linked to proteins. Thus, the linear glycan chitosan was coupled by amide bond formation between its aminogroups and a carboxylic acid functionalized PEG (Prego et al. 2006). *N*-hydroxysuccinimide (NHS) esters are frequently used for derivatization of primary amino groups. A heterodifunctional PEG with a protected amino group at one end and a NHS ester at the other end was used to locate active molecules at the distal end of the PEG chain linked to chitosan. An amino functionalized α-mannoside was introduced in this way (Fernandez-Megia et al. 2007). Chitosan partially amidated with lactobionic acid was PEGylated for DNA carrier studies (Park et al. 2001). These PEG-chitosan copolymers have shown improved biocompatibility. A natural fructan was coupled to linear amino-PEGs and the bioactivity was studied (Lin et al. 2010). Specific PEGylation of the carbohydrate of ricin A-chain was accomplished by periodate oxidation followed by hydrazide formation with a hydrazine derivative of PEG (Youn et al. 2005). The method was applied to proteins previously glycosylated with oligosaccharides (Salmaso et al. 2008). Also, a protein was modified by sequential enzymatic transfer of galactose and PEGylated sialic acid from the respective nucleotides (DeFrees et al. 2006). However, few reports describe direct PEGylation of small sugars. Diels-Alder chemistry was used to immobilize several different monosaccharides bearing a cyclopentadiene group, attached through a PEG linker, to a monolayer presenting benzoquinone groups on a gold surface (Houseman and Mrksich 2002). Monosaccharide-capped PEGylated quantum dots (PEG-QDs) have been prepared by reaction of the PEG-QD with a glycosidic thiol (Kikkeri et al. 2009). A recent paper describes the enzymatic copolymerization of monosaccharide acetal derivatives with PEG-600 dimethyl ester (Bhatia et al. 2011). We have previously reported the preparation and characterization of linear PEG conjugates of lactose analogs for inhibition studies on TcTS (Giorgi et al. 2010). Although the conjugates showed inhibition values similar to those of the precursor disaccharide, the stability in circulation was not improved, probably due to the low molecular weight of the polymer used. In order to increase the molecular weight together with the loading of the active compound, we used here multiarm PEGs as carriers. They have the advantage over the linear ones of presenting several sites for conjugation. We expected that placing active sites at the end of each arm, situated far away from each other, would allow independent interaction with the enzyme.

Results and discussion

Synthesis of multiarm PEG conjugates

In this study, we used commercial 8-arm polydisperse PEGs, constructed on a hexaglycerin core structure, each activated as *N*-hydroxysuccinimidyl succinate (8-arm succinimidyl succinate polyethyleneglycol, SSPEG) or with an amino group (8-arm NH_2PEG). Two different approaches were used for PEGylation of disaccharide derivatives containing β-D-Gal*p* as a non-reducing unit. (1) Amide formation between the amino group of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (1, Scheme 1) and 8-arm SSPEG. (2) Amide formation between lactobionolactone (6, Scheme 2) and 8-arm NH_2PEG. In the first approach, the sugar ring structure is conserved whereas in the second method the lactone yields an open chain derivative of the sugar on amidation.

PEGylation of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside. We have previously shown that the disaccharide β-D-galactopyranosyl-(1→6)-2-acetamido-2-deoxy-α-D-glucose as well as its benzyl glycoside were good acceptors of sialic acid in the reaction catalyzed by TcTS (Agusti et al. 2007). They were also competitive inhibitors when 3′-sialyllactose was used as the sialic acid donor and *N*-acetyllactosamine as the acceptor. Accordingly, it was shown that the methyl glycoside of β-D-Gal*p*(1→6)-D-Gal*p* is even a better substrate than lactose in the TcTS reaction (Harrison et al. 2011). Compound **1**, prepared as previously described (Giorgi et al. 2010), was conjugated by amidation of the 2-amino group with 8-arm PEGs constructed on a hexaglycerin core and activated as *N*-succinimide succinate (SSPEG) (Scheme 1). Two commercial SSPEG with molecular weights of 20 204 and 42 680 Da, according to the certificate of analysis of the manufacturer, were used. The reaction took place in buffer pH 8 at room temperature for 24 h. Using the disaccharide in excess, easily removed by dialysis, the reactive polymer was consumed and the conjugates were recovered as white powders by lyophilization. Compounds **3** and **5** were obtained in 70 and 60% yield, respectively. The conjugates react on a thin layer chromatography (TLC) plate by charring with the sulfuric acid reagent to give a black spot, whereas the original PEGs could not be detected under the same conditions. Conjugation and purity of the compounds were proved by comparing the NMR spectra of **3** and **5** with those for the starting PEGs (**2** and **4**, respectively). Three groups of signals allowed the confirmation of conjugation in every arm. (1) The methylene protons of the succinate linker (H-e, H-f, Figure 1A), which appeared as a broad singlet at δ 2.70 ppm in the starting PEG, shifted upfield as two multiplets centered at δ 2.63 ppm and δ 2.52 ppm on amidation with the sugar (H-e′ and H-f′, Figure 1B). (2) The disappearance of the signal at δ 2.80 ppm (H-s, Figure 1A), which integrated for 4 protons and corresponded to the equivalent methylene protons of the oxy-succinimide. (3). The protons of the CH_2OCO group in the conjugate (H-a′, Figure 1B) appeared as a multiplet at δ 4.27

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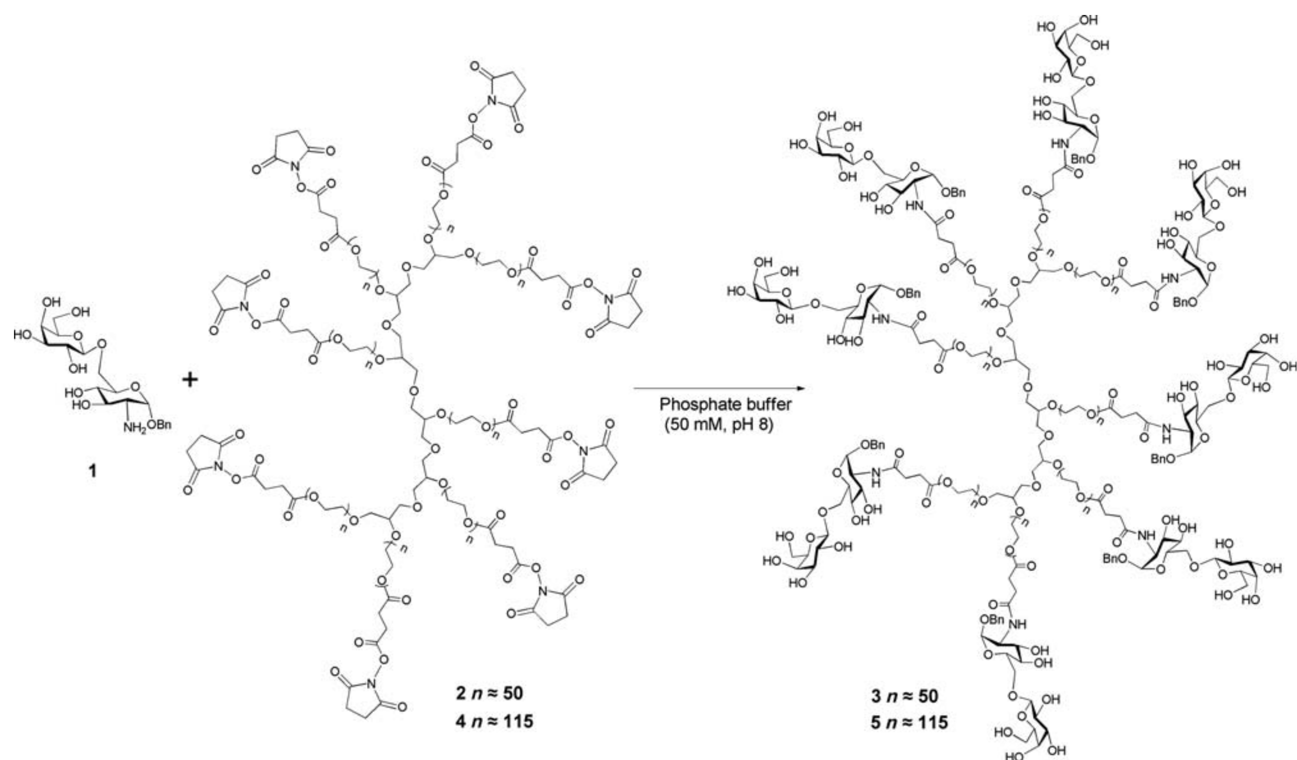
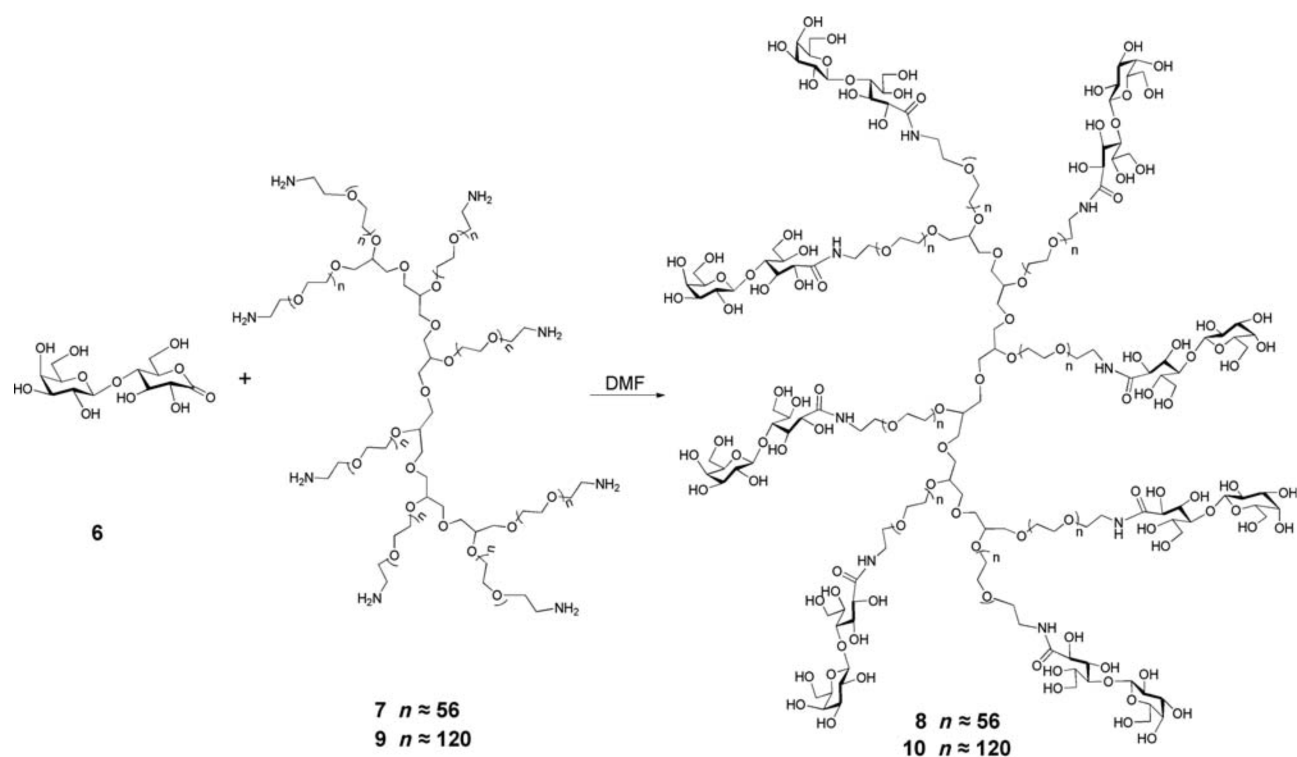


Scheme 1. Synthesis of *N*-PEGylated derivatives of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside.



Scheme 2. Lactobionolactone amidation with 8-arm NH_2PEG.

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Fig. 1. Comparison of ^{1}H NMR spectra of 8-arm SSPEG **2** (**A**) and the corresponding PEG conjugate **3** (**B**).

ppm, somewhat shifted to higher fields (δ 4.30 ppm) with respect to the underivatized PEG (H-a, Figure 1A). Accordingly, the expected integration ratio between a′ and the two sugar anomeric protons (2:1) was observed. These protons at δ 4.45 ppm ($J_{1',2'}$ = 7.8 Hz, H-1′) for β-Gal*p* and δ 4.94 ppm ($J_{1,2}$ = 3.7 Hz, H-1) for the α-glucoside unit appeared well separated from the arm-related signals. The ^{1}H NMR spectrum of **5** (not shown) was similar to that of **3**. Estimation of the number of oxyethylene groups and thus, the average molecular weight for the conjugates was calculated by integration of the corresponding signal with respect to the anomeric protons. Values of Mn 27 204 and Mn 57 080 were obtained for **3** and **5**, respectively (Table I). This overestimation with respect to the values calculated considering complete derivatization could be explained by interference of the satellite side bands as the central peak of the PEG repeating unit increases in intensity. Size exclusion chromathography (SEC) molecular weight determination, on the other hand, showed lower values than that calculated on the basis of the molecular weight of the original PEGs, probably due to the hydrodynamic volume and spherical geometry of the multiarm conjugate, different from the polystyrene standards used to calibrate the column. This feature has also been reported for other star polymers (Wolf et al. 2010).

The ^{13}C NMR spectrum of **3** (Figure 2B) also showed disappearance of the signal at δ 25.4 ppm (s, Figure 2A) for the two methylene carbons of the succinimide moiety and differentiation of the succinate methylene carbons e′ and f′ which appeared at δ 30.1 ppm and δ 29.3 ppm. The oxymethylene groups of the PEG chain (c′) appeared as a strong peak at δ 69.8 ppm together with the hexaglycerol oxymethylene carbons. Among the sugar signals, the anomeric carbons were shown at δ 103.4 ppm (β-Gal*p*) and δ 96.0 ppm (α-GlcN), and the C-2 signal of the sugar amide at δ 53.6 ppm. The amide carbonyl that links the sugar to the polymer arm appeared close to the ester carbonyl at δ 174.7 ppm.

PEGylation of lactobionolactone. We had previously determined that lactobionic acid was a good acceptor of sialic acid and inhibitor of TcTS (Agusti et al. 2004). In the present

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Table I. Molecular weights determined by ^{1}H NMR spectrometry and SEC

		Found			
	Calcd[a]	NMR[b]	SEC[c]		
Sample	M_w (g/mol)	M_n (g/mol)	M_n (g/mol)	M_w (g/mol)	PDI
3	22 732	27 204	17 250	19 693	1.14
5	45 208	57 080	27 746	30 582	1.10
8	23 256	24 536	10 924	12 668	1.16
10	45 400	54 940	21 989	25 936	1.18

[a]Determined with manufacturer data for the original PEG.
[b]Number average molecular weight (Mn) was determined by integration of the signal corresponding to the oxyethylene groups with respect to the signal for the anomeric protons.
[c]Size exclusion chromathography (SEC) was performed by high-performance liquid chromatography using a Styragel H-3 column and tetrahydrofuran (THF) as the mobile phase at a flow rate of 1.0 mL/min. Commercial polystyrene standards were used for calibration.
PDI, polydispersity index.

work, we used lactobionolactone (**6**) to form an amide linkage with two different molecular weight amino-functionalized 8-arm PEGs (**7** and **9**, Scheme 2) of 20 536 and 42 680 Da, according to the certificate of analysis of the manufacturer. The reaction was performed at 90°C in anhydrous dimethylformamide. Although the starting PEG could be revealed with ninhydrin, the products could not be detected with this reagent and were detected on a silicagel TLC plate by charring with the sulfuric acid reagent. After dialysis, conjugates **8** and **10** were recovered with 93 and 87% yields, respectively.

The ^{1}H NMR spectrum of **8** (Figure 3B) clearly showed new signals in the methylene region when compared with the original PEG (Figure 3A). Thus, the two hydrogens of the methylene linked to the nitrogen (H-a′, Figure 3B) appeared as two multiplets centered at δ 3.41 ppm and δ 3.48 ppm whereas in the underivatized PEG the corresponding hydrogens (H-a, Figure 3A) appeared as a multiplet centered at δ 3.19 ppm. The shifting of the signal for these protons to a lower field on amidation confirmed complete conjugation.

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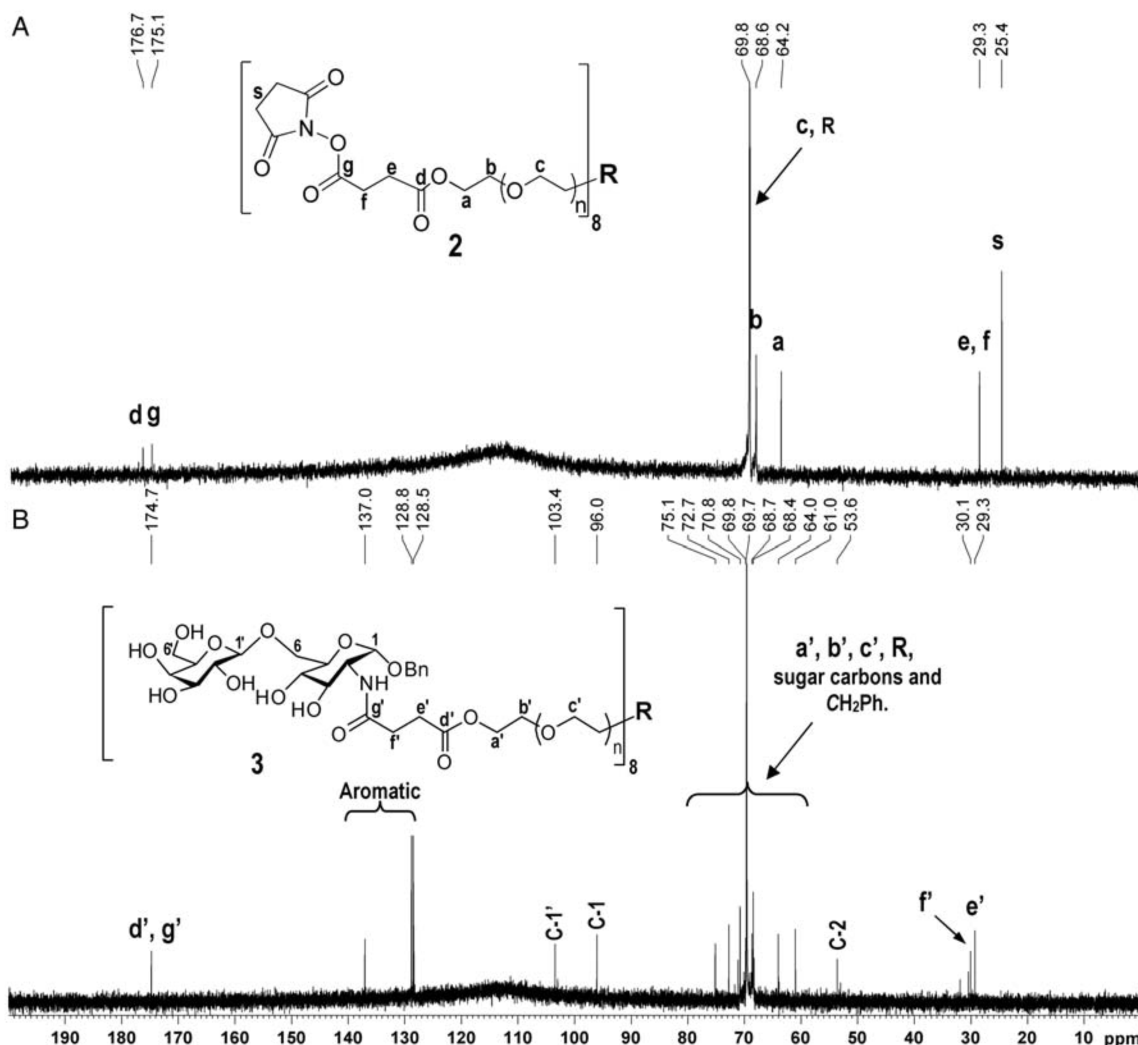

Fig. 2. Comparison of ^{13}C NMR spectra of 8-arm SSPEG **2** (**A**) and the corresponding PEG conjugate **3** (**B**).

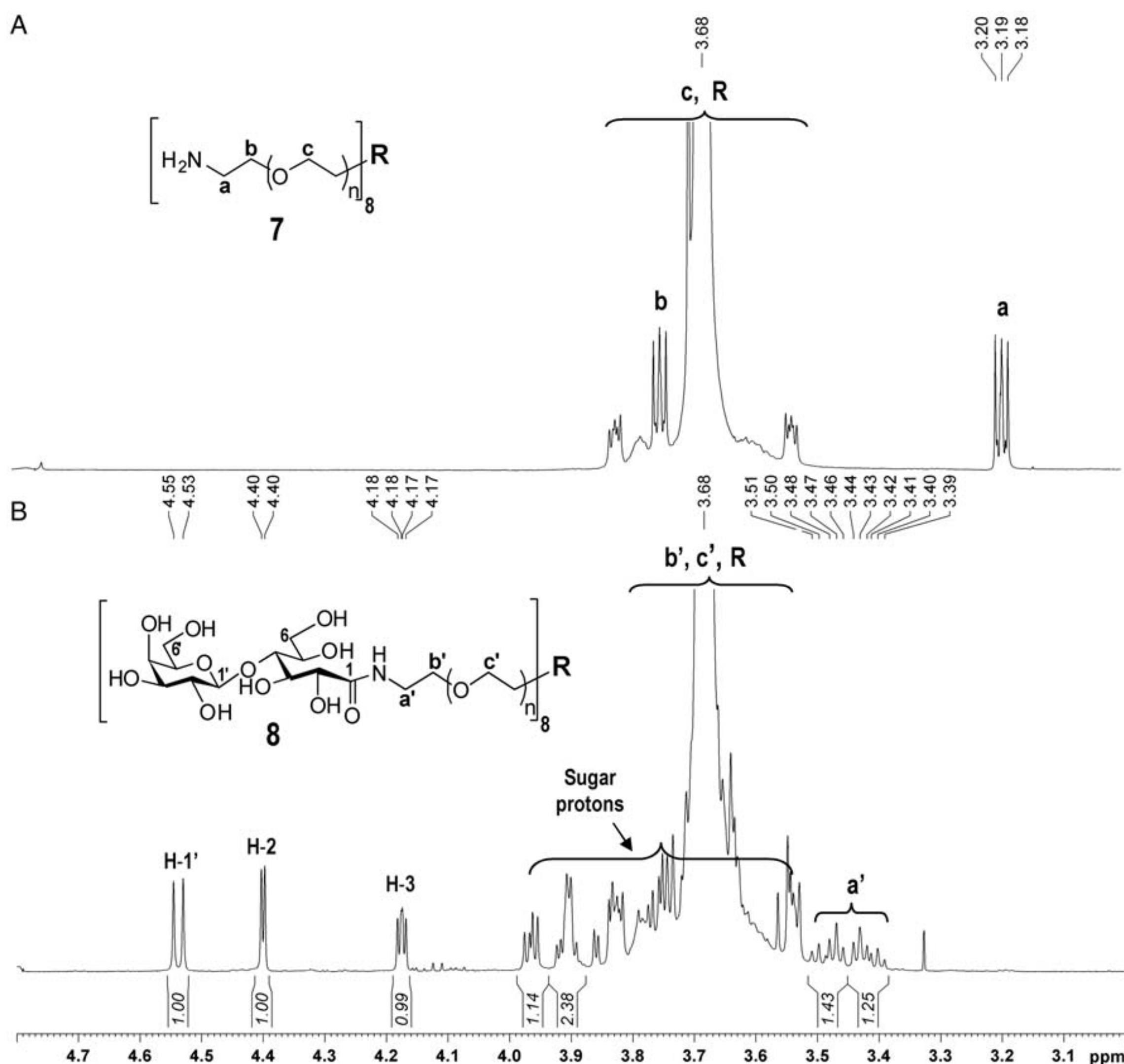

Fig. 3. Comparison of ^{1}H NMR spectra of 8-arm NH_2PEG **7** (**A**) and the corresponding PEG conjugate **8** (**B**).

The only anomeric proton, corresponding to the β-Gal unit was shown at δ 4.54 ppm as a doublet with $J = 7.8$ Hz. A characteristic doublet at δ 4.40 ppm ($J = 2.7$ Hz) due to the H-2 of the derivatized lactobionamide was also observed. The oxyethylene protons of the repeating units gave a strong signal centered at δ 3.68 ppm. A similar spectrum was observed for compound **10**. The main features in the ^{13}C NMR spectrum of **8** (Figure 4B), confirming the conjugation, are the appearance of the carbonyl signal of the amide at δ 174.4 ppm and the anomeric signal at δ 103.5 ppm (β-Gal*p*). For the PEG moiety, on the other hand, the methylene next to the amide (a′, Figure 4B) appeared at slightly higher field, δ 38.6 ppm, than in the underivatized PEG. The oxyethylene groups of the chain appeared as a strong peak at δ 69.6 ppm. As mentioned above, values of molecular weight obtained by integration of ^{1}H NMR spectra, Mn 24 536 and Mn 54 940 for **8** and **10,** respectively (Table I), were higher than calculated. Accordingly, SEC molecular weight determination showed values lower than expected.

Stability of the PEG conjugates in blood

To determine whether conjugation of the disaccharide with a multiarm PEG increased the permanence in blood with respect to the stability found for a linear 5 kDa PEG conjugate (Giorgi et al. 2010), compound **5** was injected in mice and a sample of blood was taken at different times after injection (controls were injected with equivalent amounts of underivatized PEG, **4**). Compound **5** was chosen because of the larger molecular weight of the PEG chain and the better water solubility with respect to the lactobionamide derivative. Quantification was performed using a colorimetric method previously reported for estimation of PEG in plasma (Sims and Snape 1980; Ramon et al. 2005). The concentration of compound **5** in a blood sample taken 1–2 min after injection was 11 mg/mL and diminished to half this value after 30 min (Figure 5). This value was maintained after 24 h. This result represented a high increase in stability with respect to the linear 5 kDa PEG conjugate which showed a fast elimination from blood. Only 4

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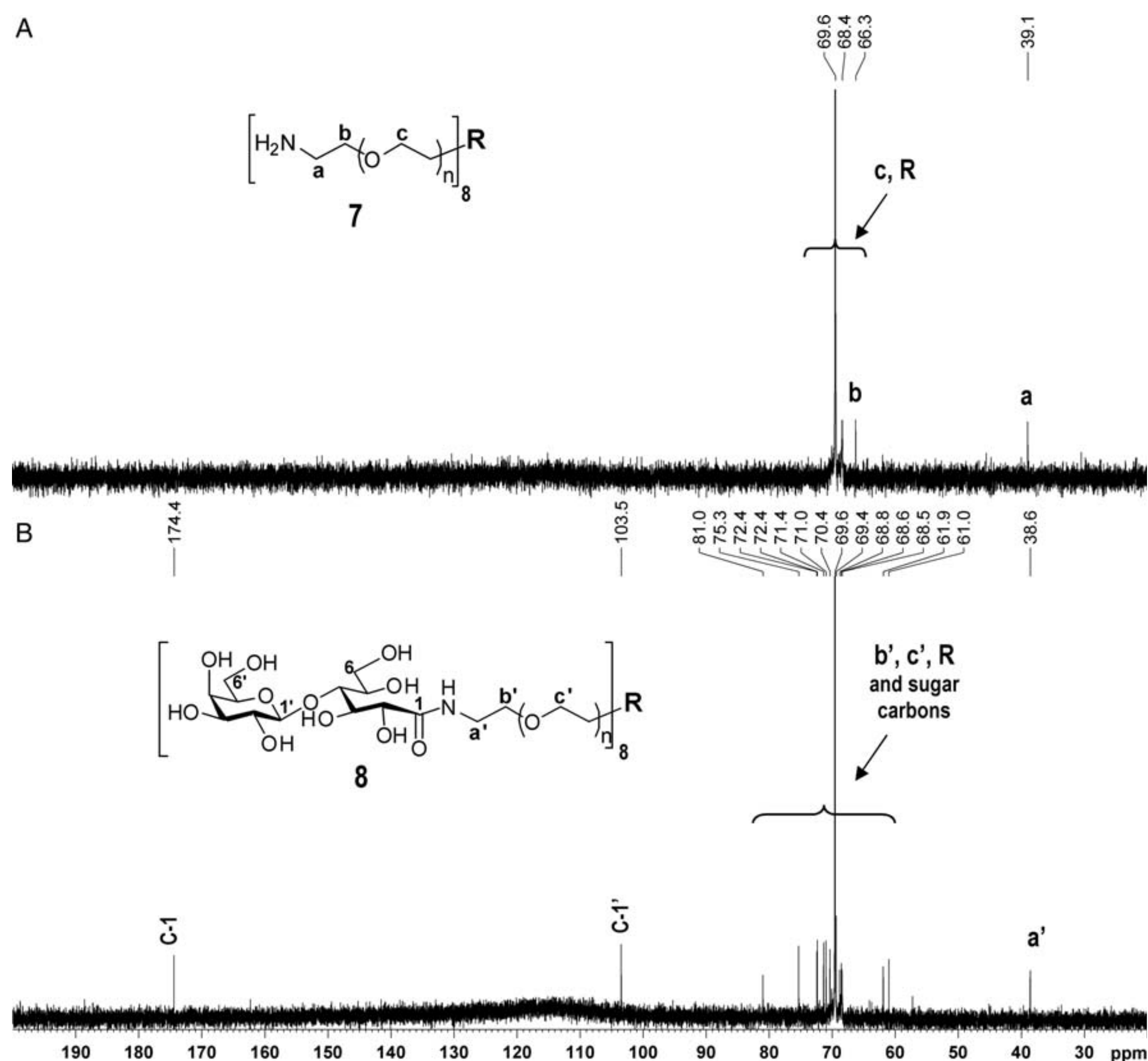

Fig. 4. Comparison of ^{13}C NMR spectra of 8-arm NH_2PEG **7** (**A**) and the corresponding PEG conjugate **8** (**B**).

mg/mL were detected when a similar amount was injected and a sample was taken a few minutes after injection and, after 30 min, decreased to 1 mg/mL. 1.26 mg of conjugate **5** was recovered in 14 µL of urine after 30 min, whereas 1.50 mg in 115 µL of urine was collected after 24 h. This suggests a constant but slow elimination by kidney filtration. Also, localization of PEG-conjugates in mice tissues cannot be ruled out.

Inhibition of sialylation by PEG-conjugates using Trypanosoma cruzi trans-*sialidase (TcTS)*

To find out whether the star PEG derivatives used in this study retain the ability of inhibiting sialylation of lactose, different concentrations of compounds **3, 5**, **8**, and **10** were tested in transfer reactions containing 1 mM of 3′-sialyllactose as a donor, 12 µM [D-glucose-1-^{14}C]-lactose as an acceptor, and TcTS (Table II). Inhibition of sialylation was quantified by measuring the radioactivity bound to an anion exchange resin after incubation and comparing with the radioactivity bound in the absence of inhibitor. The PEGylated amides (**3** and **5**) of the aminodisaccharide **1** showed IC_{50} values 6–8 times lower than the original disaccharide. Since each conjugate carries eight sugar acceptors, one may infer that each arm interacts independently with the enzyme. Also, the PEG derivatives of the open chain sugars (**8** and **10**), showed lower IC_{50} values than the sugar precursor. For all compounds, the larger conjugates were better inhibitors, suggesting that a longer chain in the PEG moiety results in a better separation of the carbohydrates and thus in a more efficient interaction with the TcTS.

Inhibition studies were also performed using mice plasma containing conjugate **5**. The competitive assay showed 80% inhibition in the first minute, about 60% at 2 h and 20% after 5 h, revealing slow diminution with time (Figure 6).

As previously reported, lactose analogs are good acceptors of sialic acid in the *trans*-sialidase reaction (Agusti et al. 2004). The extent of sialylation was determined by high performance anion exchange chromatography (HPAEC) and a K_M value of 0.13 mM was determined for the transfer of sialic acid from sialyllactose to compound **1** (unpublished results). We assume that the polyvalent conjugates also act by competing with the natural acceptors for the sialic acid.

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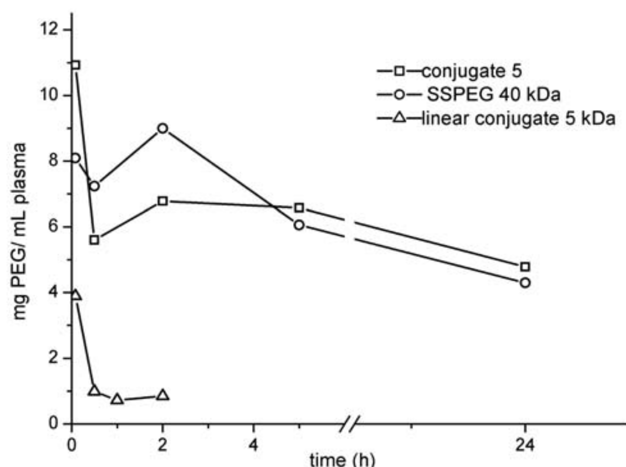

Fig. 5. Plasma clearance of PEG conjugates. Adult mice were intravenously injected with 200 µL of PBS containing 30 mg (3.3 mM) of compound **5,** or 30 mg of 8-arm SSPEG 40 kDa (**4**). A 5 kDa linear PEG conjugate (Giorgi et al. 2010) was also injected for comparison. Blood was extracted from the tail at different times, and serum was used to assay for free or combined PEG using a colorimetric assay.

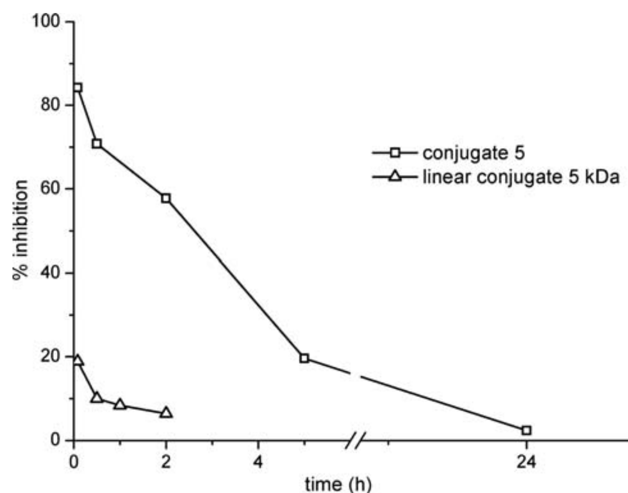

Fig. 6. Inhibition of TcTS in plasma containing PEG conjugates. Adult mice were intravenously injected with 200 µL of PBS containing 30 mg (3.3 mM) of compound **5,** or 30 mg of 8-arm SSPEG 40 kDa (**4**). A 5 kDa linear PEG conjugate was also injected for comparison. The inhibition of TcTS by the PEG conjugate present in serum was performed by adding 10 µL of serum taken at different times to a reaction mixture containing 1 mM 3′-sialyllactose, 12 µM [D-glucose-1-^{14}C] lactose (55 mCi/mmol) and 0.5 ng of purified TcTS enzyme in 30 µL of 20 mM HEPES-Na (pH 7.5), 0.2% BSA, 30 mM NaCl. After 60 min at room temperature, QAE-Sephadex was added, negatively charged compounds were eluted with 800 µL of 1 M NaCl and radioactivity was measured in a liquid scintillation counter. Results are expressed in percentage of inhibition of *trans*-sialidase activity. Radioactivity measured without addition of serum was considered as 0% of inhibition (100% of enzymatic activity).

Table II. TcTS inhibition by lactose analogs and their PEGylated derivatives using the radioactive inhibition assay

Inhibitor	IC_{50} (mM)
Lactitol	0.21
Lactobionic Acid	0.33
1	0.70
3	0.12
5	0.08
8	0.21
10	0.09

Trans-sialidase activity was measured as the transfer of sialic acid from 1 mM 3′-sialyllactose to 12 µM [D-glucose-1-^{14}C] lactose (55 mCi/mmol) in the presence or absence of increasing concentrations of inhibitor (0.01 to 1.5 mM) and using 0.5 ng of purified TcTS enzyme in 30 µL of 20 mM HEPES-Na (pH 7.5), 0.2% BSA, 30 mM NaCl. After 60 min at room temperature, the reaction was stopped by dilution with 1 mL of water. QAE-Sephadex was added and the resin was washed twice with water. Negatively charged compounds were eluted with 800 µL of 1 M NaCl and quantified in a liquid scintillation counter. Results are expressed in percentage of inhibition of *trans*-sialidase activity. Radioactivity measured without addition of any inhibitor was considered as 0% of inhibition (100% of enzymatic activity).

Experimental section

Materials and methods

Eight-arm PEG succinimidylsuccinate with a hexaglycerin core (R) (8-arm SSPEG 20 kDa and 8-arm SSPEG 40 kDa. The certificate of analysis provided by the manufacturer gave molecular weights of 20 204 and 42 680 Da, respectively) and eight-arm PEG amine with a hexaglycerin core (R) (8-arm NH_2PEG 20 kDa and 8-arm NH_2PEG 40 kDa. Analysis gave molecular weights of 20 536 and 42 680 Da, respectively) were purchased from Jenkem Technology (Beijing, China). Lactobionic acid, lactose and 3′sialyllactose were purchased from Sigma Chemical Co. [D-Glucose-1-^{14}C]-lactose was purchased from Amersham Biosciences. Benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) was prepared as previously reported (Giorgi et al. 2010). NMR spectra were recorded with a Bruker AM 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 30°C. Molecular weight determination by SEC was performed with a high performance liquid chromatography Waters System (Waters 600 pump with a Waters 2414 refractive index detector) using Styragel H-3 column (Waters). The calibration was performed with commercial polystyrene standards. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1.0 mL/min. Analytical TLC was performed on 0.2 mm Silica Gel 60 F254 (Merck) aluminium supported plates. Detection was effected by spraying with 10% (v/v) sulfuric acid in ethanol containing 0.5% *p*-anisaldehyde and charring or, for amino-compounds, by spraying with 0.2% ninhydrin in acetone and charring. Radioactivity was measured on WinSpectral 1414 liquid scintillation counter (Wallac).

*Conjugation of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) with 8-arm SSPEG 20 kDa (**2**)*

A solution of compound **1** (35 mg, 0.08 mmol) and 8-arm SSPEG 20 kDa (**2**, 100 mg, 0.005 mmol) in 1.5 mL phosphate buffer (50 mM, pH 8) was left at room temperature for 24 h. The reaction was monitored by TLC (EtOH:H_2O, 8:2). Starting compound **1** had R_f 0.6 whereas its PEGylated conjugate **3** appeared as a black spot, closed to the origin, by

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spraying with the sulfuric acid reagent and heat charring. Unreacted PEG is not detected with this reagent. Excess of disaccharide and salts were removed by dialysis against water (cut off 3500). After lyophilization, compound **3** (81 mg, 71%) was obtained as a white powder. ^{1}H NMR (D_2O): δ 7.76 (m, 5H, aromatic), 4.94 (d, $J = 3.6$ Hz, H-1), 4.80 and 4.58 (2d, 2H, $J = 11.8$ Hz, PhCH_2), 4.45 (d, $J = 7.8$ Hz, H-1′), 4.27 (m, 2H, H-a′), 4.17 (d, 1H, $J = 9.6$ Hz, H-6), 3.96–3.90 (m, 4H, sugar protons), 3.88–3.55 (m, H-b′, H-c′, hexaglycerin core and sugar protons), 2.63 (m, 2H, H-e′), 2.52 (m, 2H, H-f′). ^{13}C NMR (D_2O): δ 174.7 (CO, d′, g′), 137.0, 128.8, 128.5 (aromatic), 103.4 (C-1′), 96.0 (C-1), 75.1, 72.7, 70.8, 69.8, 69.7, 68.7, 68.4, 64.0, 61.0, (hexaglycerin core and sugar carbons, PhCH_2, c′, b′, a′), 53.6 (C-2), 30.1 (f′), 29.3 (e′).

*Conjugation of benzyl β-D-galactopyranosyl-(1→6)-2-amino-2-deoxy-α-D-glucopyranoside (**1**) with 8-arm SSPEG 40 kDa (**4**)*

Compound **1** (40 mg, 0.093 mmol) was conjugated with 8-arm SSPEG 40 kDa (**4**, 200 mg, 0.005 mmol) and the reaction processed as described for **3**. Conjugate **5** (130 mg, 60%) was obtained as a white powder. ^{1}H NMR (D_2O): δ 7.46 (m, 5H, aromatic), 4.94 (d, $J = 3.6$ Hz, H-1), 4.80 and 4.58 (2d, 2H, $J = 12$ Hz, PhCH_2), 4.45 (d, $J = 7.8$ Hz, H-1′), 4.27 (m, 2H, H-a′), 4.17 (d, 1H, $J = 9.5$ Hz, H-6), 3.96–3.90 (m, 4H, sugar protons), 3.88–3.55 (m, H-b′, H-c′, hexaglycerin core and sugar protons), 2.63 (m, 2H, H-e′), 2.55 (m, 2H, H-f′).

Conjugation of lactobionolactone (6) with 8-arm NH_2 PEG 20 kDa (7)

Lactobionolactone (**6**) was prepared from lactobionic acid as previously described (Isbell and Frush 1963). Compound **6** (80 mg, 0.24 mmol) and 8-arm NH_2 PEG 20 kDa (**7**, 100 mg, 0.005 mmol) were dissolved in anhydrous dimethylformamide (2 mL) under argon. Triethylamine (10 µL) was added to pH 8 and the reaction mixture was heated at 90°C. The reaction was monitored by TLC, detecting with ninhydrin the starting NH_2 PEG close to the origin and the sugar lactone with the sulfuric acid reagent ($R_f = 0.57$, EtOH:H_2O:AcOH, 16:1:3). After 6 h, no product was detected with ninhydrin and the PEGylated lactobionamide was detected at the origin with the sulfuric acid reagent. Excess of lactobionolactone was removed by dialysis against water (cut off 3500). After lyophilization, compound **8** (106 mg, 93%) was obtained as a white powder. ^{1}H NMR (D_2O): δ 4.54 (d, 1H, $J = 7.8$ Hz, H-1′), 4.40 (d, 1H, $J = 2.7$ Hz, H-2), 4.17 (m, 1H, H-3), 3.96 (dd, 1H, $J = 4.0$ Hz, $J = 6.5$ Hz, H-4), 3.93–3.88 (m, sugar protons), 3.88–3.52 (m, H-b′, H-c′, hexaglycerin core and sugar protons), 3.48 and 3.41 (m, 2H, H-a′). ^{13}C NMR: δ 174.4 (C-1), 103.5 (C-1′), 81.0, 75.3, 72.4, 72.3, 71.4, 71.0, 70.4, 69.6, 69.4, 68.8, 68.6, 68.5, 61.9, 61.0 (b′, c′, hexaglycerin core and sugar carbons), 38.6 (a′).

*Conjugation of lactobionolactone (6) with 8-arm NH_2 PEG 40 kDa (**9**)*

Compound **6** (80 mg, 0.24 mmol) was dissolved in anhydrous dimethylformamide (1 mL) under argon and 8-arm NH_2 PEG 40 kDa (**9**, 60 mg, 0.0014 mmol) was added in two steps. The reaction mixture was heated at 90°C for 6 h. By this time no ninhydrin reacting product was detected. After dialysis and lyophilization, compound **10** was obtained as a white powder (55.7 mg, 87.3%). ^{1}H NMR (D_2O): δ 4.57 (d, 1H, $J = 7.8$ Hz, H-1′), 4.43 (d, 1H, $J = 2.7$ Hz, H-2), 4.21 (m, 1H, H-3), 4.0 (m, 1H, H-4), 3.99–3.90 (m, 3H, sugar protons), 3.90–3.55 (m, H-b′, H-c′, hexaglycerin core and sugar protons), 3.50 and 3.46 (m, 2H, H- a′).

Stability of PEG conjugates in blood

Adult mice (C3H strain) were intravenously injected with 200 µL of phosphate buffered saline (PBS) containing 30 mg (3.3 mM) of compound **5,** or 30 mg of 8-arm SSPEG 40 kDa (**4**) or an equivalent amount of a 5 kDa PEG linear conjugate (Giorgi et al. 2010). Blood was extracted from the tail at different times, and serum was used in assaying free or combined PEG. Urine was also collected at different times for analysis of PEG conjugates. A colorimetric assay for PEG in plasma protein fractions (Sims and Snape 1980), later adapted for analysis of PEGylated interferon was used (Ramon et al. 2005).

Competitive radioactive assay for trans-*sialidase activity*

Trans-sialidase activity was measured as the transfer of sialic acid from 1 mM 3′-sialyllactose to 12 µM [D-glucose-1-^{14}C] lactose (55 mCi/mmol) by 0.5 ng of purified TcTS enzyme in 30 µL of 20 mM Hepes-Na (pH 7.5), 0.2% bovine serum albumin, 30 mM NaCl. After 60 min at room temperature, the reaction was stopped by dilution with 1 mL of water. QAE-Sephadex (Amersham Pharmacia Biotech) was added and the resin was washed twice with water. Negatively charged compounds were eluted with 800 µL of 1 M NaCl and quantified in a WinSpectral 1414 liquid scintillation counter. When required, the purified enzyme was diluted in the reaction buffer before use (0.5 ng of TcTS611/2 rendered about 4000 cpm per hour).

The effectiveness of the PEGylated sugars as inhibitors was assayed by adding increasing concentrations (0.01–1.5 mM) of them to a standard reaction mixture. The inhibition of TcTS by the PEG conjugate present in mice serum was performed by adding 10 µL of serum to a standard reaction mixture. In both cases the potential inhibitors were preincubated with the enzyme for 20 min at room temperature. Results are expressed in percentage of inhibition of *trans*-sialidase activity. Radioactivity measured without addition of any inhibitor was considered as 0% of inhibition (100% of enzymatic activity).

Concluding remarks

Multiarm (star) PEG derivatization has been successfully used to improve the efficacy of lactose derivatives as inhibitors of the *trans*-sialidase from *T. cruzi*. The sugars were conjugated with the end-functionalized PEGs in a simple reaction. The higher stability in blood showed by these star-PEG conjugates encourages in vivo studies on the inhibition of TcTS from chagasic patients. Further studies on tissue distribution would shed more light to understanding drug action. The PEG conjugates are also potentially useful as inhibitors of clinically relevant receptors of β-galactoside-binding proteins. In this

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respect, lactose-containing dendrimers and amino derivatives of lactulose have been prepared and their binding properties have been described (André et al. 1999; Rabinovich et al. 2006). On the other hand, the sialylated conjugates, being soluble, may be recognized by sialic acid-binding proteins in biological systems. Our current efforts are directed to scale-up the synthesis of the star conjugates and their *trans*-sialylation products in order to determine the extent of sialylation and to perform further biological studies.

Funding

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), National Research Council (CONICET) and Universidad de Buenos Aires. The work of A. C. C. Frasch was partially supported by International Research Scholar grants from the Howard Hughes Medical Institute. A. C. C. Frasch, R. M. de Lederkremer and R. Agustí are research members of CONICET.

Conflict of interest

None declared.

Abbreviations

HPAEC, high performance anion exchange chromatography; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PEG, polyethyleneglycol; PEG-QDs, PEGylated quantum dots; SEC, size exclusion chromatography; SSPEG, succinimidyl succinate polyethyleneglycol; TcTS, *Trypanosoma cruzi trans*-sialidase; THF, tetrahydrofuran; TLC, thin layer chromatography.

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