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Polysialic acids: potential in drug delivery

G. Gregoriadis[a], B. McCormack[a], Z. Wang[a] and R. Lifely[b]

[a]*Centre for Drug Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK* and [b]*The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, UK*

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A number of bacterial polysialic acids were injected intravenously into mice. Half-lives (up to 40 h) in the blood circulation were dependent on the polysialic acid used, increased by deacylation of their phospholipid moiety, decreased with shorter chain derivatives and appeared to be dose independent. A model drug (fluorescein) covalently coupled to a polysialic acid was found to assume the half-life of its carrier. Results suggest that intact or deacylated polysialic acids and shorter chain derivatives can be used to augment the half-lives of drugs, small peptides, proteins and drug delivery systems in the blood circulation, thus prolonging their pharmacological action.

Polysaccharide; Polysialic acid; Drug delivery system; Drug targeting; Drug clearance

1. INTRODUCTION

There are many instances where optimal use of drugs requires their extended presence within the vascular system or in extravascular areas [1]. For example, some antibiotics and cytostatics and a variety of peptides and proteins including hormones, cytokines, enzymes, antibodies and haemoglobin (as a blood surrogate) are excreted or removed from the circulation rapidly and before therapeutic concentrations in target areas can be achieved. Such drugs may be more effective, less toxic and also used in smaller quantities if their presence in the blood circulation (and hence interaction with corresponding receptors or substrates intravascularly or extravascularly) could be prolonged [2,3]. Similarly, increased half-lives of drug delivery systems such as liposomes [4], other colloidal systems [5], polymers [5] and antibodies [6] would facilitate targeting of drugs to cells other than those (e.g. the reticuloendothelial system; RES) by which many of these systems are normally intercepted [4,5].

Attempts to increase the half-lives of a number of short-lived proteins have been made successfully by coating these with low molecular weight (750–5,000) monomethoxypoly(ethyleneglycol) (mPEG). Liposomes and polystyrene microspheres coated with mPEG [7–10] or poloxamers [11,12] have also exhibited increased half-lives. It has been suggested [3] that mPEG action in prolonging the circulation time of proteins and particles is due to the formation of a shell of mPEG molecules around their surface which sterically hinders interaction with factors responsible for their clearance.

Here we report on an alternative type of macromolecules which could serve to increase the circulation time not only of small peptides and conventional drugs (a role which low molecular weight mPEG, known [3] to be excreted rapidly through the kidneys, is unlikely to fulfill) but also of larger proteins, other biopolymers and particles. Such macromolecules are the naturally occurring polymers of *N*-acetyl neuraminic acid (NeuNAc) (referred to here as polysialic acids) and include the serogroup B capsular polysaccharide from *Neisseria meningitidis* B and *Escherichia coli* K1, the serogroup C capsular polysaccharide C from *N. meningitidis* C, and the polysaccharide K92 from *E. coli* K92 (Fig. 1) as well as their shorter chain derivatives. The highly hydrophilic nature of polysialic acids (Fig. 1) and the absence of a known receptor in the body for NeuNAc suggested [13] that polysialic acids may exhibit long half-lives in the blood circulation. Results show that half-lives (up to 40 h) in the blood circulation of intravenously injected mice depend on the polysialic acid used, can be further increased by deacylation of the phospholipid moiety, decrease with shorter chain derivatives of a given polysialic acid and appear to be dose independent. Further, a model drug, fluorescein, covalently linked to a polysialic acid assumes the half-life of the latter. It is proposed that intact and deacylated polysialic acids as well as shorter chain derivatives can be used to augment the half-lives of drugs, peptides, proteins and drug delivery systems (to which polysialic acids are linked) thus prolonging their pharmacological action. A preliminary account of this work has been presented elsewhere [14].

Correspondence address: G. Gregoriadis, Centre for Drug Delivery Research, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK.

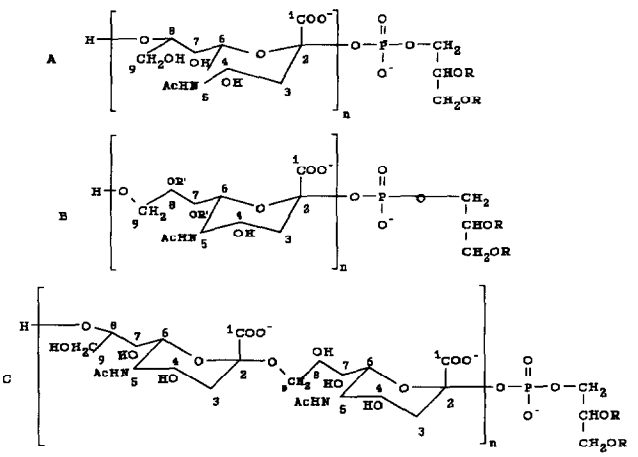

Fig. 1. (A) Serogroup B capsular polysialic acid B (PSB) from *N. meningitidis* or *E. coli* K1 is a homopolymer (n = 199) of α-(2–8)-linked *N*-acetyl neuraminic acid. (B) Serogroup C capsular polysialic acid (PSC) from *N. meningitidis* C is a homopolymer (n = 74) of α-(2–9)-linked *N*-acetyl neuraminic acid; R′: H or Ac. (C) Polysialic acid (PSK92) from *E. coli* K92 is a heteropolymer (n = 78) of alternate units of α-(2–8)-α-(2–9)-linked *N*-acetyl neuraminic acid. All three polysialic acids contain a phospholipid molecule covalently linked to the reducing end of the polymers.

2. MATERIALS AND METHODS

N. meningitidis B polysaccharide B (PSB) (average chain length 199 NeuNAc units), *N. meningitidis* C polysaccharide C (PSC) (average chain length 74 NeuNAc units) and *E. coli* K92 polysaccharide (PSK92) (average chain length 78 NeuNAc units) were prepared as previously described [15,16]. Their degree of acylation was determined [17] and found to be 66%, 20% and 37%, respectively. Fluorescein as such (sodium salt) or in its isothiocyanate form (FITC) were from Sigma (London). All other reagents were of analytical grade.

2.1. *Deacylation of the phospholipid moiety of polysialic acids*

Solutions of PSB, low molecular weight PSB (82 NeuNAc units), PSC and PSK92 in 0.1 M NaOH (typically 10 mg/ml) were incubated at 37°C for 4 h [17] and subsequently dialyzed exhaustively against phosphate-buffered saline (Buffer) composed of 0.44 mM sodium phosphate, 2.7 mM potassium chloride and 0.14 M sodium chloride, pH 7.4. Deacylated polysialic acids were used within 24 h of their preparation.

2.2. *Preparation of the polysialic acid–fluorescein conjugate*

Fluorescein was covalently coupled to PSB as described previously [18]. Briefly, PSB was dissolved in dimethylsulphoxide as the tetrabutyl ammonium salt and stirred in the presence of FITC for 24 h at room temperature. Uncoupled FITC was removed by sequential dialysis against 0.1 M ammonium acetate, pH 9, precipitation of the PSB with 3 vols. of ethanol, followed by gel filtration through Sephadex G-25 and a second ethanol precipitation step before freeze-drying. PSB had a degree of substitution of 0.009 (i.e. 9 molecules of dye per 10^3 molecules PSB) and an average chain length of 82 NeuNAc units [17]. The PSB–fluorescein conjugate was radiolabelled with ^{125}I as previously described [19].

2.3. *Assay of polymeric* N-*acetyl neuraminic acid*

Diluted blood samples (see later) were centrifuged at 3,000 rpm for 10 min to remove blood cells. Supernatants containing the plasma were mixed with trichloroacetic acid (8% final concentration), kept at 4°C for 1 h and then centrifuged at 3,000 rpm for 10 min to precipitate native plasma protein-bound NeuNAc. Supernatants as well as other polysialic acid samples were assayed for NeuNAc by the method of Svennerholm [20]. Estimation of NeuNAc was carried out on the basis of standard curves made with each of the polysialic acids used, appropriately diluted either in Buffer or in blood plasma from intact mice. In preliminary work it was established that NeuNAc values in the blood plasma (after trichloroacetic acid precipitation) of mice taken before treatment were nil.

2.4. *Assay of fluorescein*

Fluorescein in diluted plasma samples (see later) was assayed fluorometrically at excitation and emission wavelengths of 482 and 512 nm, respectively. Values were estimated from a standard curve made with appropriately diluted plasma containing varying amounts of added dye.

2.5. *Animal experiments*

Male T.O. (Clinical Research Centre) mice weighing 25–30 g were injected into the tail vein with 0.10–0.25 ml Buffer containing PSB, PSC or PSK92 (1–2 mg per mouse) either in their intact or fully deacylated form. In some experiments mice were injected with 0.25 ml Buffer containing various amounts of low molecular weight PSB covalently coupled to ^{125}I-labelled fluorescein, or fluorescein only. Animals were bled from the tail vein at time intervals and blood (typically 50 μl) was placed into 0.5 ml Buffer for the same assay of NeuNAc, ^{125}I-radioactivity or fluorescein.

3. RESULTS AND DISCUSSION

3.1. *Polysialic acid clearance from the circulation*

The clearance pattern of PSB from the blood circulation of injected mice was biphasic with 50% of the dose removed 3 min after injection (Fig. 2). The remainder assumed a linear rate of clearance with a half-life of 20 h. PSB, as well as PSC and PSK92, in solution have a phospholipid moiety covalently attached through its phosphate group to their reducing end [21] (Fig. 1). As a result, polysialic acids exhibit micellar behaviour and form aggregates [21]. The PSB used here was partially deacylated (see section 2) probably because of long-term storage, and only the acylated remainder would be expected to form aggregates. This would explain the partial rapid loss of PSB from the circulation (Fig. 2). Hydrolysis of the acyl groups (e.g. by alkali treatment) abolishes the slightly hydrophobic nature of the acylated molecules, leads to their deaggregation and perhaps a slower rate of clearance. Fig. 2 shows that this is indeed the case; only 5–10% of the fully deacylated PSB was removed rapidly, the remainder exhibiting a linear rate of clearance with a half-life of 30 h. As in the case of PSB, clearance of PSC, was initially rapid with nearly 70% of the injected dose removed within 3 min (Fig. 3) even though PSC was only 20% acylated (see section 2). Thereafter, removal was slower. Surprisingly, a considerable amount (about 50%) of the fully deacylated PSC was initially also cleared rapidly, the remainder assuming a linear pattern with a half-life of 20 h (Fig. 3). It should be noted, however, that in the conditions of deacylation there is a concomitant hydrolysis of the *O*-acetyl groups of carbons 7 and/or 8 (Fig. 1) of PSC, an event which could have contributed to the

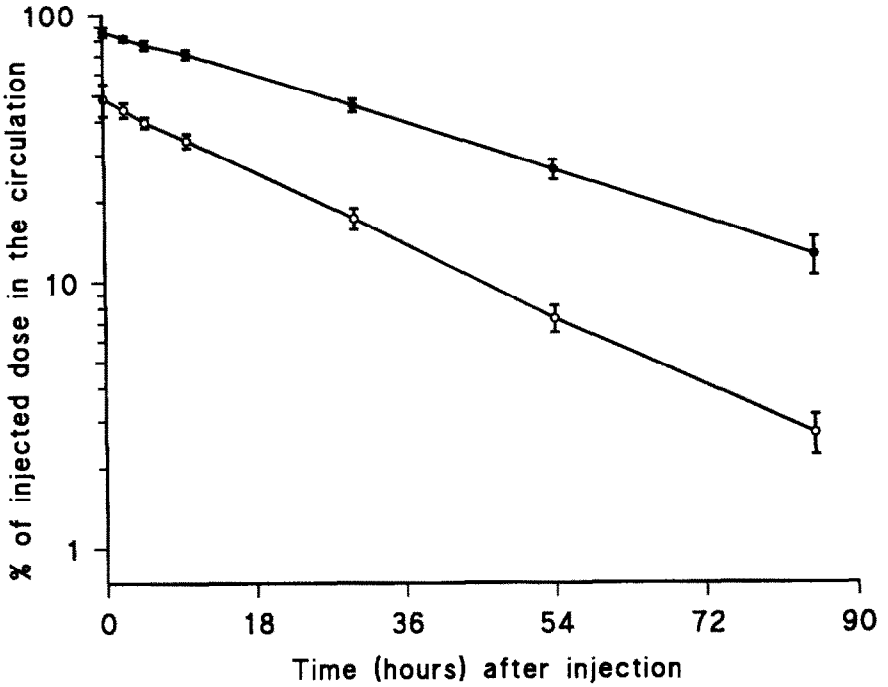

Fig. 2. Clearance of PSB from the blood circulation. In six separate experiments, mice in groups of 3–4 animals were injected intravenously with 1.1–2.0 mg of intact (○) or deacylated (●) PSB and bled at time intervals. NeuNAc in the blood plasma samples was assayed as described in section 2 and expressed as % ± S.D. of the dose in total blood. (Values from all groups treated with intact and deacylated PSB, respectively, were pooled.) Blood volume was estimated as 7% of the body weight [9]. For other details see the text.

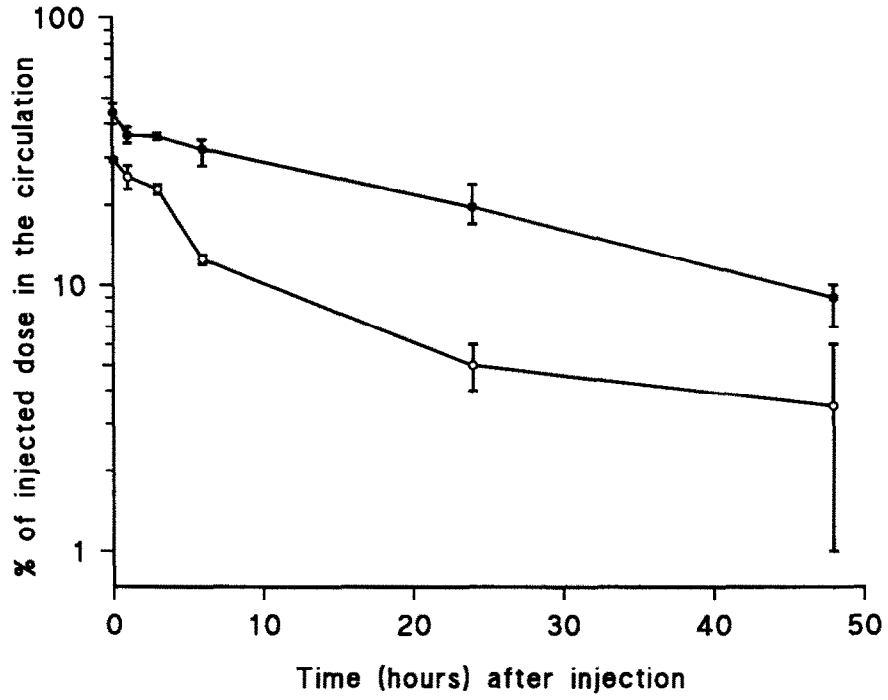

Fig. 3. Clearance of PSC from the blood circulation. Mice in two groups of 5 were injected intravenously with 2 mg of intact (○) or deacylated (●) PSC. For other details see legend to Fig. 1.

rapid clearance of deacylated PSC. On the other hand, there was no apparent difference in the clearance patterns of PSK92 before and after full deacylation (Fig. 4). Following a relatively slow clearance during the first 6 h, patterns became linear, with half-lives of 40 h. Similar results were observed in a second identical experiment performed with the same batch of PSK92 (data not shown).

Results in Figs. 2–4 thus suggest that the rate of removal of a given polysialic acid from the circulation is dependent on the presence or absence of phospholipid acyl groups. It also appears to depend on the structure of polysialic acids, since the α-(2–9)-linked PSC is cleared more rapidly than the α-(2–8)-linked PSB or the α-(2–8)–α-(2–9)-linked PSK92. Further as the chain lengths of the three polysialic acids are an average, the preparations are polydisperse. Therefore, the presence of lower molecular weight moieties may contribute to the early rapid removal of much of the polysialic acid from the circulation, particularly for PSC which has a lower average chain length (74) than PSB (199 NeuNAc units). In this respect, experiments with PSB of short chain length (15 NeuNAc units) have revealed (data not shown) that over 90% of the injected dose is removed from the circulation within 30 min. This, however, is not sufficient to explain the intriguing result with PSK92, which has an average chain length (78 NeuNAc units) no higher than PSC, yet is not removed rapidly from circulation, whether intact or deacylated. It suggests that factors other than chain length and aggregation of polysialic acids are also important in determining circulatory half-life.

3.2. *Clearance of a model drug bound to polysialic acid*

Prolonged circulation of the polysialic acids studied, prompted us to investigate the extent to which a model drug (fluorescein) covalently coupled to polysialic acid, a low molecular weight deacylated PSB (82 NeuNAc units), would assume the half-life of its carrier. Data in Fig. 5 show that clearance of coupled fluorescein (which, as such, is removed from the circulation very rapidly (Fig. 5), is independent of the dose of injected PSB for the amounts tested. Following the removal of about 80% of the dose within 2.5 h, the remainder exhibited a half-life of 5 h, presumably that of the conjugate. Since the limit of sensitivity of the NeuNAc assay in the blood plasma would not allow the monitoring of NeuNAc values for doses lower than 0.5 mg PSB per mouse in this experiment, only NeuNAc values from mice injected with the higher doses of deacylated PSB are shown (Fig. 5). These values are similar to those corresponding to conjugated fluorescein, further supporting the view that fluorescein is cleared in conjunction with its carrier. A similar half-life (5.5 h) was obtained with the intact PSB–FITC conjugate (results not shown), probably because the proportion of molecules of the low molecular weight intact PSB (derived from the larger molecular weight PSB; 199 NeuNAc units)

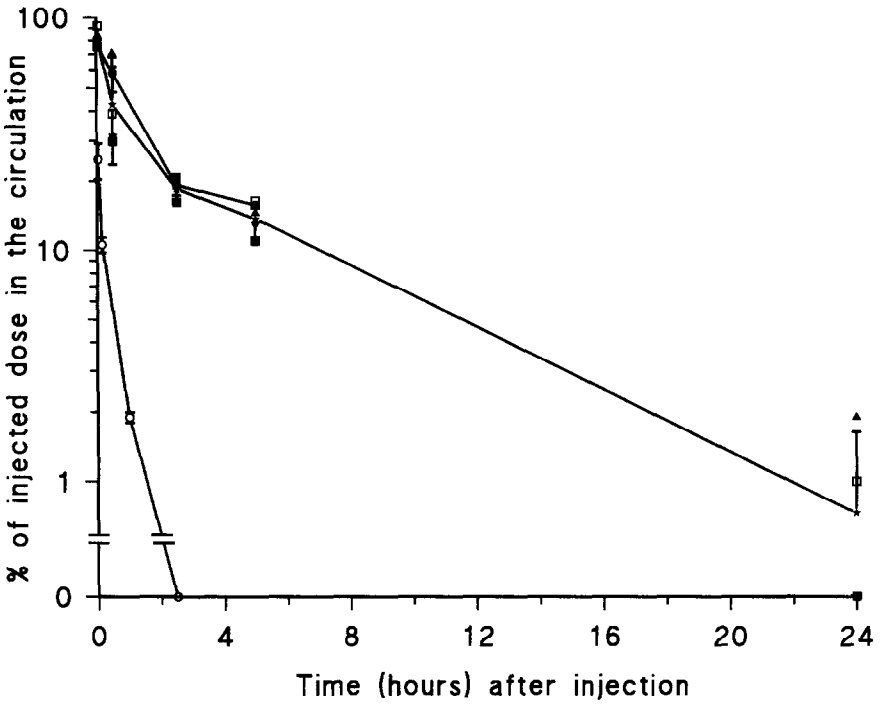

Fig. 4. Clearance of PSK92 from the blood circulation. Mice in two groups of 4 were injected intravenously with 1.8 mg of intact (○) or deacylated (●) PSK92. For other details see legend to Fig. 1.

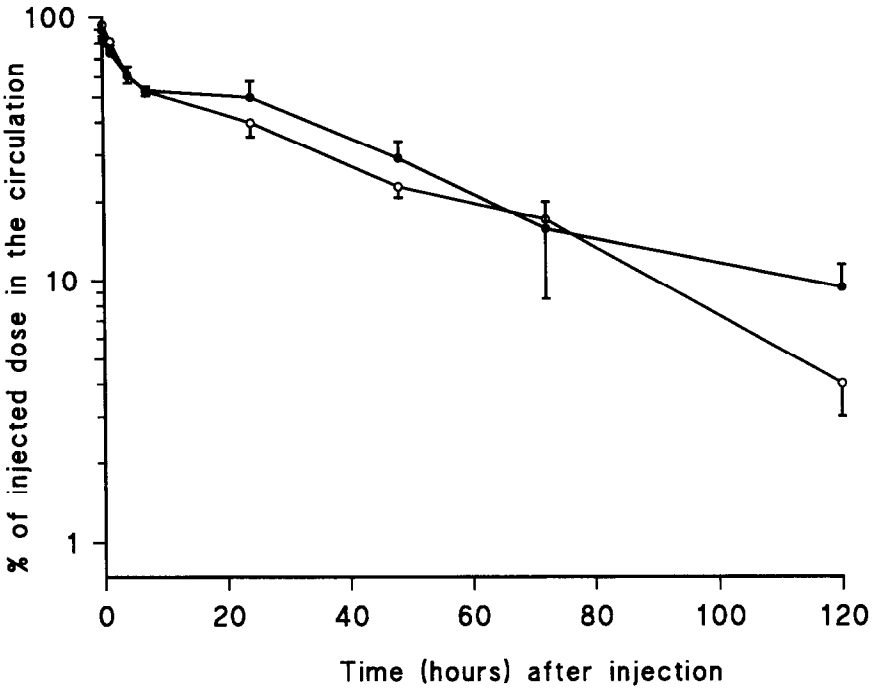

Fig. 5. Clearance of low molecular weight fluorescein–PSB conjugate from the blood circulation. Mice in groups of three were injected intravenously with 28 (▼), 102 (■), 510 (▲) and 1,528 μg (●) of PSB conjugated to [^{125}I]fluorescein or with 40 μg fluorescein only (○). Values are means ± S.D. of ^{125}I-radioactivity (closed symbols), NeuNAc (□) or fluorescein. Stars denote the mean of ^{125}I mean values for all doses at each time interval. For other details see legend to Fig. 1.

that would contain a phospholipid moiety would be too low to contribute to significant aggregation and a shorter half-life.

In conclusion, polysialic acids such as those described here, could potentially retain rapidly cleared drugs within the vascular and extravascular areas for prolonged periods of time. Because of the dependence of clearance rates of polysialic acids not only on the type used and the state of their phospholipid (intact or deacylated), but also on the molecular size (compare Figs. 2 and 5), it would be possible to tailor clearance rates of drugs to satisfy specific needs. It is envisaged that large molecular weight polysialic acids would be suitable for the delivery of one or more molecules of small molecular weight drugs and peptides. Shorter chain polysialic acids, on the other hand (derived by autohydrolysis of long-chain molecules), could be used to coat large proteins as well as drug delivery systems such as liposomes. Conjugation of drugs and liposomes to polysialic acids could be carried out by a variety of methods, depending on the reactive groups available on the interacting entities. Possible sites of conjugation in polysialic acids include the non-reducing end which, on periodate oxidation, would generate a reactive aldehyde, the carboxyl and hydroxyl groups, and the amino groups becoming available on deacetylation (Fig. 1). However, caution is required as coupling reactions could potentially damage the tertiary structure of the longer chain polysialic acid and thus, possibly, alter their clearance patterns.

Polysialic acids have the advantage of being biodegradable and catabolic products (e.g. NeuNAc) are not known to be toxic. Furthermore, polysialic acids, like other polysaccharides, are T-independent antigens and do not induce immunological memory. For instance, PSB is non-immunogenic in animals and humans which has hampered attempts to produce a vaccine against *N. meningitidis* group B or *E. coli* K1 [22]. Although PSC and PSK92 have been shown to be immunogenic in humans, it is necessary to use polysaccharides with molecular weights in excess of 50,000 Da (average chain length greater than 170 NeuNAc units) [21]. This, however, is not true when considering polysialic acids coupled to proteins. Here, polysialic acids can become T-cell dependent antigens with induction of memory, and no restriction on the size of the polymer applies. Nonetheless, immune responses are difficult to achieve (especially for PSB) although immunogenic vaccines have been manufactured by coupling polysialic acids to some protein carriers [18,22,24]. Another, perhaps more important, consideration in selecting a polysialic acid as a drug carrier, is antigenicity (i.e. binding of the antigen to its antibodies). Although it has been shown that antibodies against some of the polysialic acid structures examined here exist at low levels in circulation, they are generally of low affinity, especially against α-(2–8)-

linked PSB [25]. Indeed, α-(2–8)-linked sialic acid structures are known to be present on host cell surfaces, thereby limiting any immunological response [26]. Finally, taking into account the pathogenicity of *N. meningitidis* it would be easier, from the practical point of view, to produce polysialic acids from slightly or non-pathogenic bacteria. Since PSB (deacylated) and PSK92 exhibit the longest half-lives (Figs. 2 and 4) and can be derived from the slightly pathogenic *E. coli* K1 (PSB) and the non-pathogenic *E. coli* K92 (PSK92) bacteria, these materials and their hydrolysis lower molecular weight products should be adopted for conjugation to drugs and drug delivery systems.

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