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Polysialic Acids: Potential Role in Therapeutic Constructs

GREGORY GREGORIADIS*, ANA FERNANDES, BRENDA McCORMACK, MALINI MITAL AND XIAOQIN ZHANG

Centre for Drug Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London, WC1N 1AX, UK

Introduction

The extended presence of drugs either within the vascular system or in extravascular use is often a prerequisite for their optimal use (Gregoriadis *et al.*, 1994). Many antibiotics and cytostatics for instance, as well as a variety of therapeutic peptides and proteins, are removed from the circulation prematurely and before effective concentrations in target tissues can be achieved. It follows that such drugs could be more effective, less toxic and also used in smaller quantities if their presence intravascularly or extravascularly (and hence interaction with corresponding receptors or substrates) could be prolonged (Lee *et al.*, 1995). Similarly, prolonged circulation of drug delivery systems such as liposomes (Gregoriadis, 1995), other colloidal systems (Davis *et al.*, 1984) and polymers (Domb *et al.*, 1997) 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 (Gregoriadis, 1995; Lee *et al.*, 1995).

To that end, the half-lives of a number of short-lived proteins (*e.g.* enzymes, cytokines, etc) have been successfully augmented (Nucci *et al.*, 1991) by conjugating these to low molecular weight (750–5,000) mono-methoxypoly(ethyleneglycol) (mPEG). Liposomes and polystyrene microspheres coated with mPEG or poloxamers are also known to exhibit increased half-lives (e.g. Senior *et al.*, 1991; Davis *et al.*, 1984). It appears that mPEG molecules prolong the circulation time of proteins and particles by forming a shell around their surface, thus sterically hindering interaction with factors responsible for their clearance (Torchilin and Papisov, 1994). However, because of their low molecular weight (which results in rapid excretion through the kidneys), such mPEG polymers are not suitable for prolonging the half-life of small therapeutic agents (e.g. small peptides and conventional drugs). Recently, we have reported (Gregoriadis *et al.*, 1993) on an alternative type of macromolecules which

*To whom correspondence may be addressed.

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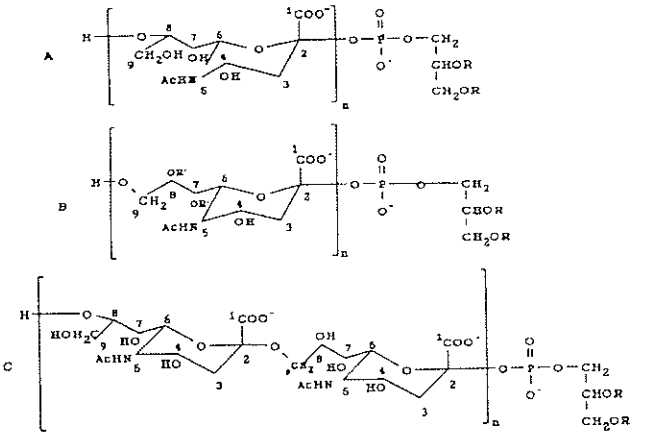

Figure 1. Structures of polysialic acids. (A) Serogroup B capsular polysialic acid B (PSB) from *N. meningitidis* or *E. coli* K1 is a homopolymer (n = 199) of α-(2–8)-linked *N*-acetyl neuraminic acid. (B) Serogroup C capsular polysialic acid (PSC) from *N. meningitidis* C is a homopolymer (n = 74) of α-(2–9)-linked *N*-acetyl neuraminic acid. (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. From Gregoriadis *et al.* (1993), with permission.

may serve to increase the half-life not only of small molecules but also of large proteins, other large biopolymers, and microparticles such as liposomes. These macromolecules are naturally occurring polysaccharides, namely polymers of N-acetyl neuraminic acid (NeuNAc) (polysialic acids). They include (*Figure 1*) the serogroup B capsular polysaccharide from *Neisseria meningitidis* B and *Escherichia coli* KI, the serogroup C capsular polysaccharide C from *N. meningitidis* C, and the polysaccharide K92 from *E. coli* K92, as well as shorter chain derivatives thereof.

Clearance of polysialic acids from the circulation

It was thought (Gregoriadis *et al.*, 1993) that because of their highly hydrophilic nature and the absence of a known receptor in the body for NeuNAc, polysialic acids would be likely to circulate in the blood for prolonged periods after intravenous injection and could, thus, serve as carriers of short lived drugs or peptides. Experiments were therefore carried out in which mice were injected with a variety of polysialic acids. NeuNAc levels in blood plasma were measured by an assay modified (Gregoriadis *et al.*, 1993) to exclude the NeuNAc of plasma glycoproteins. Results showed that the clearance pattern of polysaccharide B (PSB) (see structure in *Figure 1*) from the blood circulation was biphasic with 50% of the dose removed 3 min after injection (*Figure 2*). The remainder of the dose assumed a linear rate of clearance with a half-life of 20 h. As *Figure 1* illustrates, PSB, polysaccharide C (PSC) and polysaccharide K92 (PSK92) have a phospholipid moiety covalently attached through its phosphate group to their reducing end. As a result, polysialic acids in solution exhibit micellar behaviour and form aggregates (Gotschlich *et al.*, 1981). The phospholipid moiety of the PSB used here was probably partially deacylated because

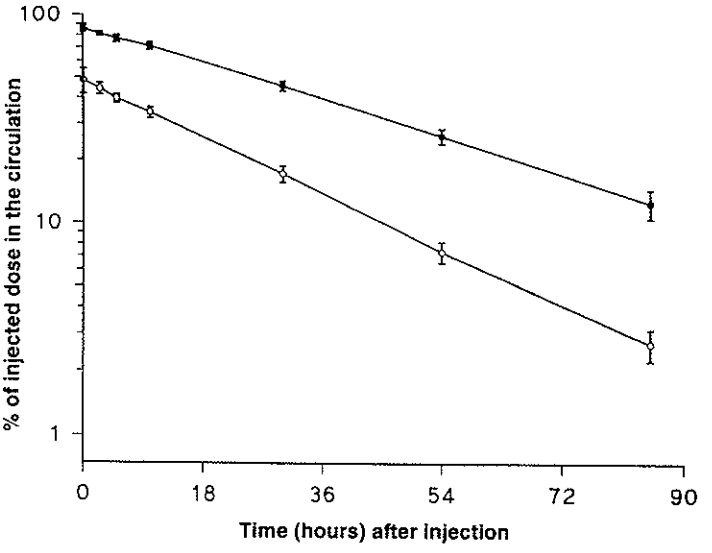

Figure 2. Clearance of PSB from the blood circulation. In six separate experiments, mice in groups of 3–4 animals were injected intravenously with 1.1–2.0 mg of intact (○) or deacylated (●) PSB and bled at time intervals. NeuNAc in the blood plasma samples was assayed as described (Gregoriadis *et al.*, 1993) 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. From Gregoriadis *et al.* (1993), with permission.

of long-term storage (Gregoriadis *et al.*, 1993), with only the acylated remainder expected to form aggregates. This would explain the rapid partial loss of PSB from the circulation, presumably in the form of aggregates. Results suggest that this is indeed the case: only 5–10% of the fully deacylated PSB was cleared from the circulation rapidly, the remainder exhibiting a linear rate of clearance with a half-life of 30 h (*Figure 2*). On the other hand, there was no apparent difference in the clearance patterns of PSK92 before and after deacylation (*Figure 3*). Following a relatively slow clearance during the first 6 h, patterns became linear with half-lives of 40 h (*Figure 3*).

It is therefore apparent that the rate of removal of a given polysialic acid from the circulation may be dependent on the presence or absence of phospholipid acyl groups. However, since the α-(2–8)-linked PSB is cleared more rapidly than the α-(2–8)-α-(2–9)-linked PSK92 (*Figures 2 and 3*), clearance may also depend on the structure of polysialic acids. Moreover, as the chain length of polysialic acids is an average and preparations are, therefore, polydisperse, low molecular weight polysialic acids may also contribute to the early rapid removal of some of the injected material from the circulation (as observed for PSB and, to a lesser extent, for PSK92) (*Figures 2 and 3* respectively). Indeed, experiments with a PSB of short chain length (15 NeuNAc units) have revealed that over 90% of the injected dose is removed from the circulation within 30 min (Gregoriadis *et al.*, 1993).

Clearance of a model drug bound to polysialic acid

The finding of prolonged half-lives for the polysialic acids used here was encouraging in terms of employing these as a means to extend the half-life of small drugs. Studies

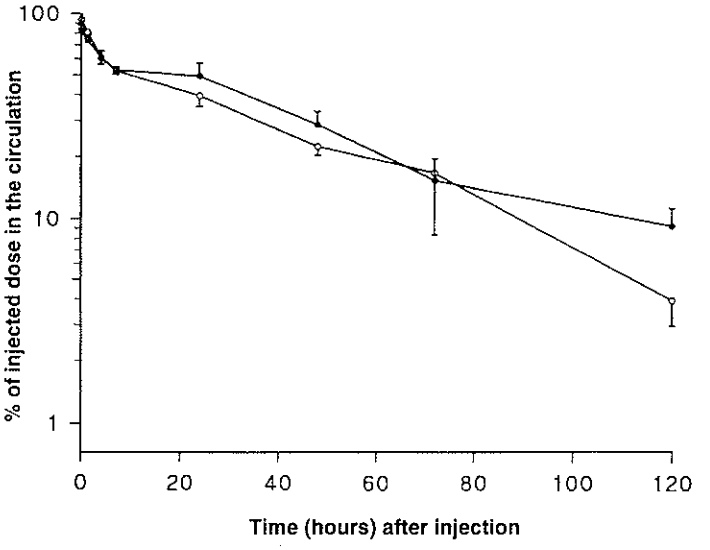

Figure 3. Clearance of PSK92 from the blood circulation. Mice in two groups of 4 were injected intravenously with 1.8 mg of intact (○) or deacylated (●) PSK92. For other details see legend to *Figure 2.* From Gregoriadis *et al.* (1993), with permission.

were therefore carried out with a model drug (fluorescein) coupled to deacylated PSB of low molecular weight (82 NeuNAc units). Data in *Figure 4* indicate that whereas fluorescein as such was removed from the circulation very rapidly, clearance of the polysialic acid-bound dye was slower and, also, 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 of fluorescein exhibited a half-life of 5 h, presumably that of the conjugate.

It is thus apparent that large molecular weight polysialic acids such as those described, could potentially retain rapidly cleared drugs and small peptides within the vascular and extravascular areas for extended periods of time. Also, because of the dependence of polysialic acid clearance not only on the type used and the presence or absence of the acyl groups of the phospholipid moiety but also the molecular size (Gregoriadis *et al.,* 1993), it would be possible to tailor clearance rates of drugs and peptides to satisfy specific needs. It is envisaged, for instance, that large molecular weight polysialic acids would be suitable for the delivery of one or more molecules (per molecule of polysialic acid) of low molecular weight drugs and peptides. On the other hand, shorter chain polysialic acids derived by the hydrolysis of long-chain molecules, could serve as a coat of large proteins as well as drug delivery systems such as liposomes (*Figure 5*). A variety of techniques could be used to conjugate polysialic acids to drugs and liposomes, depending on the reactive groups available on the interacting entities. Possible sites (*Figure 1*) of conjugation in polysialic acids include the non-reducing end which, on periodate oxidation, generates a reactive aldehyde, the carboxyl and hydroxyl groups, and the amino groups becoming available on deacetylation (Gregoriadis *et al.*, 1993). However, caution is required as coupling reactions could potentially damage the tertiary structure of the longer chain polysialic acids and alter their patterns of clearance.

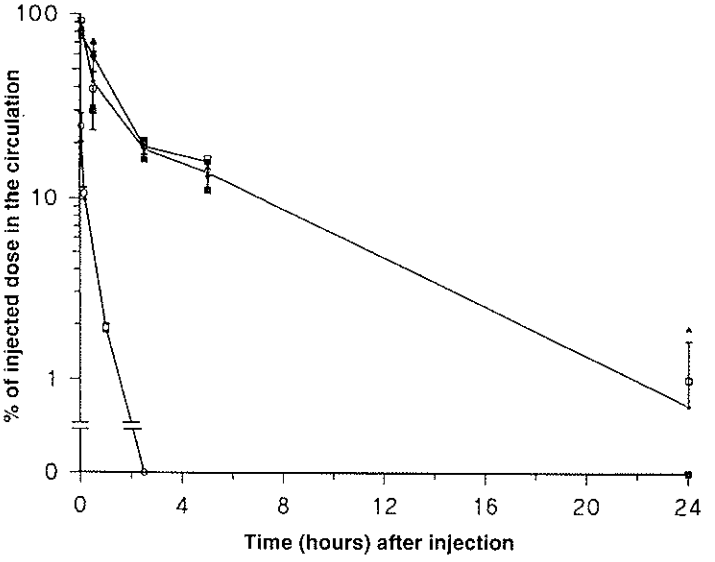

Figure 4. Clearance of low molecular weight fluorescein-PSB conjugate from the blood circulation. Mice in groups of three were injected intravenously with 28 (▽), 102 (■), 510 (△) and 1,528 μg (●) of PSB conjugated to [^{125}I]fluorescein or with 40 μg fluorescein only (○). Values are means ± S.D. of ^{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 *Figure 2*. From Gregoriadis *et al.* (1993), with permission.

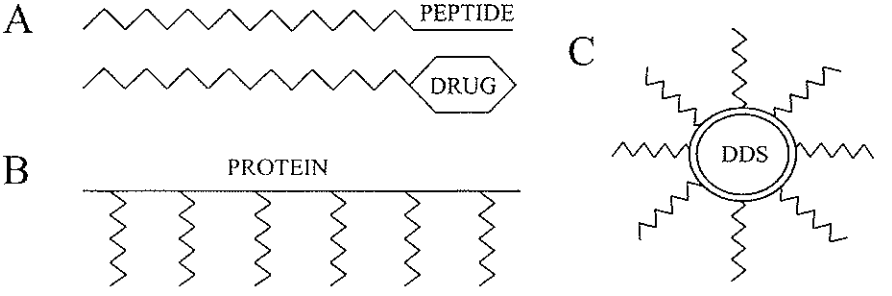

Figure 5. Schemes of polysialic acid use in drug delivery. Long polysialic acids can be used to prolong the circulation time of small drugs and peptides.(A) Shorter polysialic acids bound to the surface of proteins (B) or liposomes (C) will render them more hydrophilic and extend their half-lives.

Polysialylated enzymes: characterization and fate *in vivo*

Therapeutic use of proteins can be hampered by proteolytic degradation and short half-lives in the circulation (Nucci *et al.*, 1991). Further, administration of large amounts of protein in order to maintain therapeutic efficacy, can often lead to toxicity and also promote adverse immune responses. As discussed elsewhere (Fernandes and Gregoriadis, 1996; Nucci *et al.*, 1991), such problems can be circumvented by the covalent coupling of proteins to hydrophilic macromolecules such as dextrans and mPEG.

The latter is by far the most successful and comprehensively studied and its use has now been extended to liposomes and other particulate systems (Lasic and Martin, 1995).

We have employed a low molecular weight polysialic acid, colominic acid (CA) (*Figure 6*), as a means to render two enzymes, namely catalase and asparaginase, more hydrophilic (Fernandes and Gregoriadis, 1996, 1997). It was anticipated that polysialylation of the proteins would not only improve their pharmacokinetics and stability, but also reduce their immunogenicity. The choice of catalase as a model therapeutic protein was based on its increasing use as an oxygen radical scavenger or in enzyme replacement therapy (Scott *et al.*, 1991). Asparaginase on the other hand, catalyses the hydrolysis of the non-essential amino-acid L-asparagine to L-aspartic acid and ammonia. As certain tumour cells that are deficient in L-asparagine synthetase (Haskell *et al.*, 1969) depend on the external supply of L-asparagine, asparaginase is currently in clinical use for the treatment of acute lymphoblastic leukaemia (Keating *et al.*, 1993). The enzyme is also active against non-Hodgkin's lymphoma and pancreatic carcinoma (Yunis *et al.*, 1977). Moreover, because normal cells produce the synthetase and are therefore not affected by the treatment, asparaginase therapy is highly selective.

POLYSIALYLATED CATALASE

Previous work (Fernandes and Gregoriadis, 1996) in which catalase was polysialylated with colominic acid following periodate oxidation of the latter at the non-reducing end (carbon 7) and subsequent coupling to the enzyme (ε amino groups) by reductive amination, showed that the extent of polysialylation was modest (3.8±0.4 moles of CA per mole of catalase). Polysialylated catalase, however, retained 70% of its initial activity at the end of the coupling reaction compared with values of 29–39% for enzyme controls treated similarly in the absence of CA and reagents. Formation of sialylated catalase was confirmed by ammonium sulphate or trichloroacetic acid precipitation, molecular sieve chromatography and SDS-PAGE electrophoresis (Fernandes and Gregoriadis, 1996). Sialylated catalase was much more stable in the presence of specific proteinases, especially chymotrypsin, than the native enzyme (Fernandes and Gregoriadis, 1996).

POLYSIALYLATED ASPARAGINASE

Earlier attempts to increase the half-life of asparaginase in the blood circulation, included entrapment into liposomes (Neerunjun and Gregoriadis, 1976) or erythrocytes (Kravtzoff *et al.*, 1990), and covalent coupling to mPEG (Cao *et al.*, 1990). The latter approach, however, led to substantial loss of enzyme activity.

Activation of colominic acid

Oxidation (activation) of CA was carried out with 0.1M sodium periodate (10 mg CA/ml periodate solution) at 20°C in the dark and in the presence of ethylene glycol to expend excess periodate. Following extensive dialysis at 4°C against ammonium carbonate solution, the preparation was freeze-dried and kept at –40°C until further use (Fernandes and Gregoriadis, 1996, 1997).

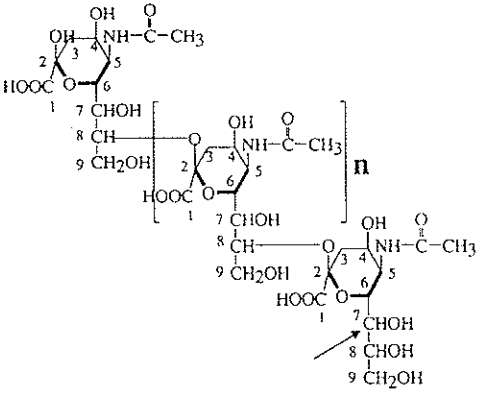

Figure 6. Structure of colominic acid. *N*-acetylneuraminic acid units are linked via α-(2→8) glycosidic linkages. Arrow indicates the carbon atom (C_7) at the non-reducing end of the sugar where periodate oxidation introduces an aldehyde group. From Fernandes and Gregoriadis (1996), with permission.

Preparation of asparaginase-colominic acid conjugates

Asparaginase (previously dialyzed to remove dextrose monohydrate) was covalently coupled to the activated CA by reductive amination in the presence of $NaCNBH_3$ (Fernandes and Gregoriadis, 1997) as previously applied for catalase (Fernandes and Gregoriadis, 1996). Three different molar ratios of CA:asparaginase (50:1, 100:1 and 250:1) were used in the coupling reaction. The sialylated asparaginase formed was isolated from non-conjugated CA by ammonium sulphate precipitation. Pellets containing the conjugate were then dissolved in 0.15M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS), and extensively dialysed against the same buffer. The dialysed samples were filtered to remove insoluble material, and asparaginase activity and protein concentration in the filtrates were determined spectrophotometrically. CA bound to the enzyme was measured (Fernandes and Gregoriadis, 1997) and values were expressed as moles of CA per mole of asparaginase or as percentage of polysialylated lysine residues. Solutions containing a known amount of polysialylated asparaginase (450–475 U/mg protein) were freeze-dried and kept at 4°C until further use. Results (Fernandes and Gregoriadis, 1997) on the extent of CA coupling to asparaginase and activity retention by the polysialylated enzyme revealed that polysialylation was directly dependent on the molar ratio of CA and enzyme used in the coupling reaction, with the highest degree of polysialylation (8.1 ± 1.7 moles of CA/mole asparaginase) achieved when a 250 fold excess of CA was present in the reaction mixture. This value (8.1) corresponds to an average of 11% of the available lysine ε-amino groups (Fernandes and Gregoriadis, 1997). However, as CA is polydisperse, values of degree of polysialylation are only average.

Asparaginase conjugates produced by other methods are known to lead to severe loss of enzyme activity, for instance as much as 70–90% for pegylated asparaginase (Cao *et al.*, 1990). In contrast, the coupling procedure used here led to only a modest loss (14–18%) of initial asparaginase activity in the polysialylated enzyme (Fernandes and Gregoriadis, 1997). It thus appears that polysialylation (or perhaps the presence of CA in the reaction mixture during the coupling procedure), protects the enzyme from

inactivation: only 17% of asparaginase activity was retained by the enzyme when subjected to identical reaction conditions in the absence of CA.

Tritiation of asparaginase

Native asparaginase was tritiated as previously described (Fernandes and Gregoriadis, 1997). Following isolation of the labelled enzyme with ammonium sulphate or precipitation with trichloroacetic acid, more than 90% of the radioactivity was recovered with the enzyme pellet. Furthermore, polyacrylamide gel electrophoresis of the labelled enzyme confirmed (Fernandes and Gregoriadis, 1997) the absence of higher molecular mass enzyme species that could have formed through formaldehyde-induced methylene bridges. In additional experiments, asparaginase was simultaneously radiolabelled and coupled to CA as described (Fernandes and Gregoriadis, 1997), by employing $NaCNB[^3H]_3$ in the reaction mixture. The enzyme conjugate was then isolated and excess label removed by ammonium sulphate precipitation. In typical preparations, more than 90% of the radioactivity in the polysialylated asparaginase could be precipitated by trichloroacetic acid, indicating that reductive methylation ensured obligatory participation of most of the ^3H in the structure of the polysialylated enzyme.

Kinetics of polysialylated asparaginase

Plots (*Figure 7*) of the effect of substrate concentration on the activity of native and polysialylated asparaginase suggested a modest (but not significant; $P>0.05$) increase in the enzyme's Km value after polysialylation. Estimation of Km values according to Hanes-Woolf were 1.68×10^{-5} M for native asparaginase and 1.90×10^{-5} M, 2.15×10^{-5} M and 2.29×10^{-5} M respectively for the polysialylated constructs made with different CA to enzyme ratios (see *Figure 8*). These values are of the same order of magnitude as those reported (Howard and Carpentier, 1972) for the clinically useful asparaginases (e.g. 10^{-5} M). V_{max} values calculated from *Figure 7* and in the same order, were 0.847, 0.901, 0.910 and 0.919 μmole min^{-1} U^{-1} (Fernandes and Gregoriadis, 1997). It thus appears that covalent coupling of CA to asparaginase and regardless of its degree of polysialylation, does not affect significantly the action of the enzyme on asparagine.

The effect of plasma on asparaginase activity

The results presented in *Figure 8* indicate that polysialylated asparaginase is more stable in the presence of (mouse) plasma at 37°C than the native (non-sialylated) enzyme: whereas polysialylated asparaginase retained most (65–83%) of its initial activity after exposure to plasma for 6 h, that of the native enzyme decreased to 13.5%. In addition, retention of activity by the polysialylated asparaginase was significantly higher for the preparation with the greatest number of CA molecules (preparation C; *Figure 8*). The effect of polysialylation on the stability of asparaginase (also observed for polysialylated catalase; Fernandes and Gregoriadis, 1996) has been tentatively attributed to changes in the microenvironment of the enzyme which must have occurred by the presence of the highly hydrophilic, negatively charged CA molecules.

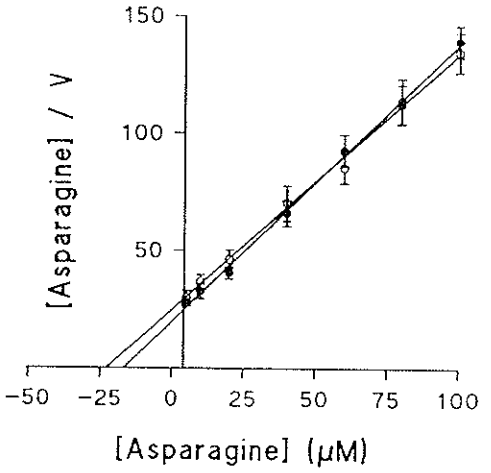

Figure 7. Hanes Woolf plots. For native (●) and polysialylated asparaginase (○). Values denote means ± S.D. (3 different experiments); V denotes velocity, expressed as μmol of liberated ammonia per min per unit (U) of enzyme. K_m values were obtained by extrapolation to the abscissa. From Fernandes and Gregoriadis (1997), with permission.

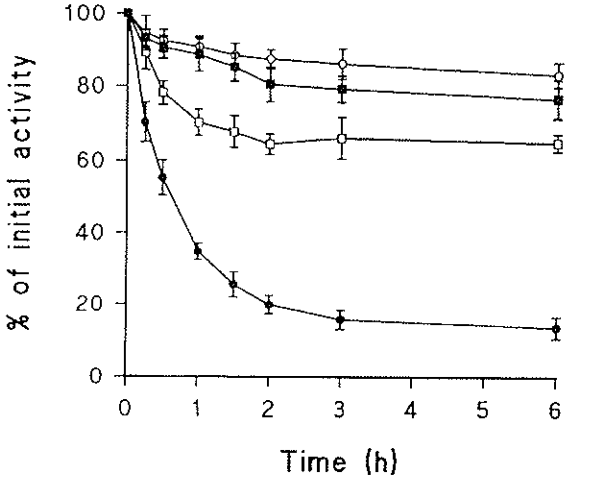

Figure 8. Retention of asparaginase activity in the presence of plasma. Native (●) and polysialylated asparaginase preparations A (□), B (■), C (○) were incubated in the presence of mouse plasma at 37°C; values denote means ± S.D. (3 different preparations). *Statistics*: results obtained at 6 h, were compared by ANOVA and *P* values corrected by the Bonferroni test. Native vs. A, B and C, $P<0.001$; A vs. B, n.s.; B vs. C, n.s.; A vs. C, $P<0.05$; n.s. = non-significant. From Fernandes and Gregoriadis (1997).

For instance, it is conceivable that a hydrophilic environment (promoted by polysialylation) combined with a shielding effect of the CA chains, contributes to a reduction in the access of plasma proteases to their target sites on the enzyme.

The effect of polysialylation on asparaginase clearance

Earlier work (results not shown) had shown that, in terms of enzyme activity, tritiation of asparaginase does not alter its pattern of clearance from the circulation. The clearance of tritiated asparaginase was therefore compared with that of the tritiated polysialylated constructs. As tritiation of the latter could only occur during the process of polysialylation, radioactivity was expected to represent polysialylated asparaginase and not intact enzyme that could also be present in the preparation. Moreover, the (radiolabelled) constructs were prepared under conditions (i.e. CA:enzyme molar ratio used in the coupling reaction) that were identical to those used for the non-radiolabelled conjugates, except that the sodium borohydride added during the reaction was tritiated.

All three constructs of polysialylated asparaginase were found to be removed from the circulation at slower rates than the native enzyme, both in terms of radioactivity (*Figure 9A*) and enzyme activity (*Figure 9B*). However, as observed with other modified enzymes (e.g. Nucci *et al.*, 1991), much of the injected dose (about 75% for native and 60–65% for polysialylated asparaginase) was removed from the circulation within 2 h after injection, the remainder exhibiting slower, linear clearance rates (*Figure 9A,B*). Moreover, on the basis of enzyme activity, terminal half-lives ($t_{1/2}\beta$) (estimated from the linear portions of asparaginase activity and ^3H radioactivity clearance patterns) were about 15 h for the native and about 38 h for the polysialylated asparaginase. Terminal half-lives were also independent of the dose injected, at least for the range of doses tested (0.5– 2.0 mg) and, for each of the polysialylated conjugates tested, similar whether derived from radioactivity or enzyme activity values. This confirmed that radioactivity measurements accurately reflected the presence of active enzyme.

Factors contributing to the clearance of injected proteins include (Delgado *et al.*, 1992) non-specific uptake by the reticuloendothelial system and receptor-mediated endocytosis by cells of the liver and other tissues where protein degradation eventually occurs. In addition, protein clearance is also influenced by molecular mass, shape and charge (Bocci, 1987; Benbough *et al.*, 1979) which determine the extent to which proteins undergo transcapillary passage or renal filtration (Bocci, 1987). In the latter case, the molecular mass cut-off is 66–70 kDa (Delgado *et al.*, 1992), i.e. well below the molecular mass (135 kDa) of asparaginase. It is likely that, at least in part, a greater resistance to plasma proteases (*Figure 8*) contributes to increases in the half-life of polysialylated asparaginase. It is also feasible that, as a result of the loss of some of the free ε-amino groups of asparaginase upon polysialylation, the modified protein is intrinsically more negatively charged. This, together with a shielding effect of the CA chains discussed above and elsewhere (Fernandes and Gregoriadis, 1996, 1997), could interfere with the interaction of the enzyme with blood and tissue components and thus curtail its recognition by tissues and removal from the circulation.

Although the reduction in the clearance rates of the polysialylated asparaginase observed in the present study is not as great as that claimed for the pegylated enzyme (e.g. Cao *et al.*, 1990), the following should be taken into consideration: (a) amounts of both native and pegylated enzyme in those studies were too low (up to 40 U per animal) compared to those (450–550 U) used in the present work and, therefore, measurements of enzyme activity may not have been as accurate, especially over

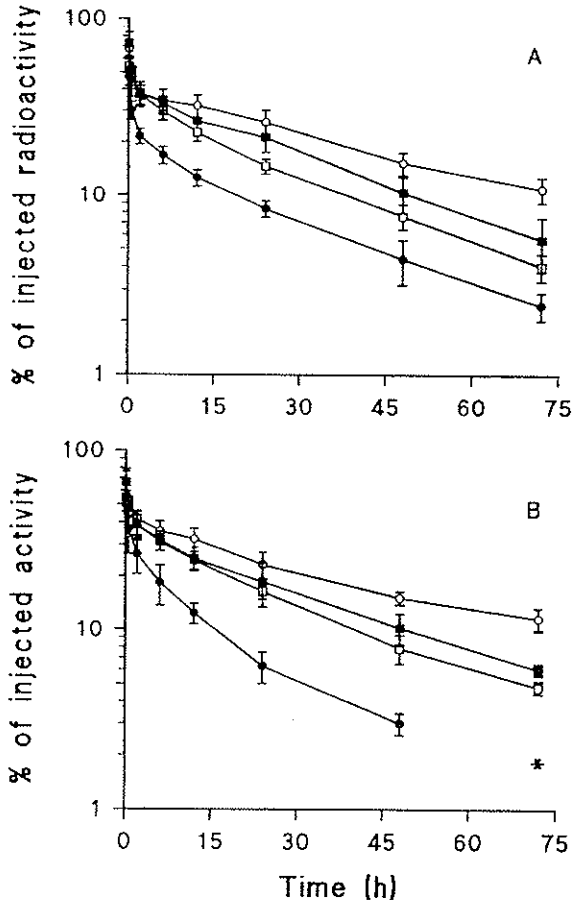

Figure 9. Clearance of asparaginase from the circulation. Mice were injected intravenously with 1 mg (550 U) tritiated native (●) and 1 mg (450-475 U) tritiated polysialylated asparaginase prepared by the use of 50:1 (□), 100:1 (■) and 250:1 (○) molar ratios of CA: asparaginase in the coupling reaction. Blood plasma obtained at time intervals was assayed for ^{3}H (A) and asparaginase activity (B). The pharmacokinetics profiles demonstrate biphasic patterns of clearance which are consistent with a two-compartment model. Values denote means ± S.D.; n=4 animals. *Native asparaginase activity was not detectable at 72 h. From Fernandes and Gregoriadis (1997).

extended periods of time. This could have in turn contributed to an overestimation of the half-lives for the pegylated asparaginase; (b) as pegylation of asparaginase leads to quantitative loss (Cao *et al.*, 1990; Uren and Ragin, 1979) of enzyme activity, polysialylation (which affects activity only modestly) (Fernandes and Gregoriadis, 1996, 1997) may be a preferred alternative as it would limit wastage of proteins; (c) to the authors' knowledge, there is no information on the fate and effect of the mPEG moiety of pegylated proteins subsequent to their uptake by tissues. Since mPEG is non-biodegradable, its accumulation intracellularly, especially on chronic use, may prove undesirable.

Immunogenicity and antigenicity of polysialic acids

In contrast to other hydrophilic macromolecules (e.g. dextran, mPEG), polysialic acids are biodegradable and their 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. PSB for instance, is non-immunogenic in animals and humans and this has hampered attempts to produce a vaccine against *N. meningitidis* group B or *E. coli* K1 (Moreno *et al.*, 1985). On the other hand, although PSC and PSK92 are 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). However, polysialic acids coupled to proteins 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. An additional, perhaps more important consideration in selecting a polysialic acid for drug or enzyme delivery, is antigenicity (i.e. binding of the antigen to its antibodies). Although antibodies against some of the polysialic acid structures do exist at low levels in circulation, they are generally of low affinity, especially those against the α-(2–8) linked structures (Mandrell and Zollinger, 1982) which are present on host cell surfaces, thereby limiting any immunological response (Finne, 1982). Finally, it would be easier from the practical point of view to produce polysialic acids from non-pathogenic bacteria (as opposed to the pathogenic *N. meningitidis*). Since PSB (deacylated) and PSK92 exhibit the longest half-lives (*Figures 2* and *3*) and can be derived from the slightly pathogenic *E. coli* K1 (PSB) or the non-pathogenic *E.coli* K92 (PSK92) bacteria, these materials and their lower molecular weight products should be adopted for conjugation to therapeutic agents or to systems that could deliver such agents.

Acknowledgements

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References

BENBOUGH, J.E.,WIBLIN, C.N., RAFTER, T.N.-A. AND LEE, J. (1979). The effect of chemical modification of L-asparaginase on its persistence in circulating blood of animals. *Biochemical Pharmacology* **28**, 833.

BOCCI, V. (1987). Metabolism of protein anticancer agents. *Pharmacological Therapeutics* **34**, 1.

CAO, S., ZHAO, Q., DING, Z., MA, L., YU, T., WANG, J., FENG, Y. AND CHENG, Y. (1990). Chemical modifications of enzyme molecules to improve their characteristics. *Annals of the New York Academy of Science* **613**, 460.

DAVIS, S.S., ILLUM, L., MCVIE, J.-G. AND TOMLINSON, E., EDS. (1984). *Microspheres and Drug Therapy*. Amsterdam: Elsevier.

DELGADO, C., FRANCIS, G.E. AND FISTER, D. (1992). The uses and properties of PEG-linked proteins. *Critical Reviews of Therapeutic Drug Carrier Systems* **9**, 249.

DOMB, A.J., KOST, J. AND WISEMAN, D.M., EDS. (1997). *Handbook of Biodegradable Polymers*. Amsterdam: Harwood Academic Publishers.

FERNANDES, A. AND GREGORIADIS, G. (1996). Synthesis, characterization and properties of sialylated catalase. *Biochimica et Biophysica Acta* **1293**, 92.

FERNANDES, A. AND GREGORIADIS, G. (1997). Polysialylated asparaginase: preparation, activity and pharmacokinetics. *Biochimica et Biophysica Acta* **1341**, 26.

FINNE, J. (1982). Occurrence of unique polysialosyl carbohydrate units in glycoproteins of developing brain. *Journal of Biological Chemistry* **257**, 11966.

GOTSCHLICH, E.C., FRASER, B.A., NISCHIMURA, O., ROBBINS, J.B. AND LIU, T.-Y. (1981). Lipid on capsular polysaccharides of gram-negative bacteria. *Journal of Biological Chemistry* **256**, 8915.

GREGORIADIS, G. (1995). Engineering liposomes for drug delivery: progress and problems. *Trends in Biotechnology* **13**, 527.

GREGORIADIS, G., MCCORMACK, B., WANG, Z. AND LIFELY, R. (1993). Polysialic acids: Potential in drug delivery. *FEBS Letters* **315**, 271.

GREGORIADIS, G., MCCORMACK, B. AND POSTE, G., EDS (1994). Liposomes *in vivo*: Control of behaviour. In *Targeting of Drugs: Advances in System Constructs*. New York: Plenum Press.

HASKELL, C.M., CANELLOS, G.P., LEVENTHAL, B.G., CARBONE, P.P. AND BLACK, J.B. (1969). L-asparaginase resistance in human leukemia-asparaginase synthetase. *Biochemical Pharmacology* **18**, 2578.

HOWARD, J.B. AND CARPENTIER, F.H. (1972). L-asparaginase from *Erwinia carotovora*. *Journal of Biological Chemistry* **247**, 1020.

KEATING, M.J., HOLMES, R., LERNER, S. AND HO, D.H. (1993). L-asparaginase and PEG asparaginas – past, present and future. *Leukemia Lymphoma* **10**, 153.

KRAVTZOFF, R., ROPERS, C., LAGUERRE, M., MUH, J.P. AND CHASSAIGNE, M. (1990). Erythrocytes as carriers for L-asparaginase. Methodological. *Journal of Pharmaceutics and Pharmacology* **42**, 473.

LEE, V.H.L., HASHIDA, M. AND MIZUSHINA, Y., EDS. (1995). *Trends and Future Perspectives in Peptide and Protein Drug Delivery*. Amsterdam: Harwood Academic Publishers.

MANDRELL, R.E. AND ZOLLINGER, W.D. (1982). Measurement of antibodies to meningococcal group B polysaccharide: low avidity binding and equilibrium binding constants. *Journal of Immunology* **129**, 2172.

MORENO, C., LIFELY, M.R. AND ESDAILE, J. (1985). Immunity and protection of mice against *Neisseria meningitidis* group B by vaccination using polysaccharide complexed with outer membrane proteins: a comparison with purified B polysaccharide. *Infection and Immunity* **47**, 527.

NEERUNJUN, D.E. AND GREGORIADIS, G. (1976). Tumour regression with liposome-entrapped asparaginase. Some immunological advantages. *Biochemical Society Transactions* **4**, 133.

NUCCI, M.L., SHORR, R. AND ABUCHOWSKI, A. (1991). The therapeutic value of poly(ethylene-glycol)-modified proteins. *Advanced Drug Delivery Reviews* **6**, 133.

SCOTT, M.D., LUBIN, B.H., ZUO, L. AND KUYPERS, F.A. (1991). Erythocyte defence against hydrogen peroxide: preeminent importance of catalase. *Journal of Laboratory Clinical Medicine* **118**, 7.

TORCHILIN, V.P. AND PAPISOV, M.I. (1994). Why do polyethylene glycol-coated liposomes circulate so long? *Journal of Liposome Research* **4**, 725.

UREN, J.R. AND RAGIN, R.C. (1979). Improvement in the therapeutic, immunological and clearance properties of *Escherichia coli* and *Erwinia carotovora* L-asparaginase by attachment of poly-DL-alanyl peptides. *Cancer Research* **39**, 1927.

YUNIS, A.A., ARIMURA, G.K. AND RUSSIN, D.J. (1977). Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. *International Journal of Cancer* **19**, 128.