Biodegradable ion-exchange microspheres based on modified polylysines

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Abstract

Poly-L-lysine was synthesized via a triethylamine initiated ring-opening polymerization of Z-L-lysine-*N*^α-carboxyanhydride, followed by deprotection of the ε-amino group. Subsequently the polylysine was sulfamated using a pyridinium-sulfate complex to obtain polymers with varying degrees of sulfamation ranging from 0 to 100%. Cytotoxicity of these materials was tested using tetrazolium metabolism (MTT) assays with B16F10 and P388 cell lines. Cytotoxicity of sulfamated polylysines with a degree of sulfamation of 80% and higher was significantly reduced as compared with the native polylysines. In both cell lines, LD₅₀ of the sulfamated materials was higher than 5 mg/ml, which was the highest dose tested. LD₅₀ of the native polylysines was lower than 0.1 mg/ml in the case of B16F10 and lower than 0.01 mg/ml in the case of P388 cells. Sulfamated polylysines with a degree of sulfamation of 80% were used to prepare microspheres (SPLMS). The microspheres were stabilized using glutaraldehyde or oxidized dextran as a crosslinking agent. The swelling ratio (defined as $V_{\text{swollen}}/V_{\text{dried}}$) of the SPLMS in aqueous media decreased with increasing ionic strength and crosslink density. The pH (ranging from 3 to 11) had no influence on the swelling ratio of SPLMS. The maximal swelling ratio was approximately 35 (SPLMS crosslinked with 0.5% glutaraldehyde in distilled water). SPLMS could be loaded with adriamycin up to a payload of 60%, which was not influenced by the crosslinking method. The adriamycin release was controlled by the ionic strength of the release medium: no drug was released in non-ionic medium such as distilled water, while 80% of the drug was released in phosphate buffered saline. This effect of the change in ionic strength could be applied to prepare a microsphere suspension in non-ionic medium such as 5% glucose solution, which does not contain free adriamycin. The drug would only be released after intra-arterial administration of this suspension, due to the presence of the blood.

Keywords: Microsphere; Ion exchange; Hydrogel; Drug delivery; Adriamycin

1. Introduction

Cytostatic agents are widely used for the treatment of cancer. In general these drugs have a narrow therapeutic window. Due to the high systemic levels of the drug needed to achieve sufficiently high drug levels at

the tumor site, severe side effects such as cardiomyopathy can occur [1,2]. A reduction of the side effects can be achieved if the drug is targeted to the site of action using microspheres [3,4]. Albumin microspheres (AMS) for instance have received much attention as possible devices for chemoembolization [5,6]. Chemoembolization refers to administration of drug-loaded particles of the requisite size (15–60 μm), to

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cause tumor infarction by occlusion or to induce transient ischemia within the tumor vasculature, whereupon the entrapped drug can be released locally [7]. However, the use of AMS for the release of adriamycin (ADR) has some drawbacks. The preparation methods afford only low payloads (1–10%), and ADR may react with the crosslinker during the stabilization process. AMS are somewhat hydrophobic and surfactants are needed to resuspend them in aqueous solutions. Another problem in the use of ADR-loaded AMS is the large burst effect usually observed. In any aqueous medium 80–90% of the drug is released within 30 min. As a result it is hard to handle the formulation in a clinical situation.

Previously, it has been demonstrated that some of these problems do not occur if microspheres are highly negatively charged and thus have ion-exchange properties. Sulfonated polystyrene ion-exchange microspheres can be loaded with ADR up to payloads of 35% and the drug release is controlled by the ionic strength of the medium [8]. In vivo testing in a liver tumor-bearing rat model showed that the toxicity of the microsphere-formulated drug was decreased as compared with the free drug, thus improving the therapeutic index [9]. A problem with these sulfonated polystyrene microspheres is that they are not degraded in the body. Upon repeated treatments accumulation of foreign material in the liver of the patient could eventually result in adverse effects. If biodegradable, biocompatible ion-exchange microspheres are used, however, this may not be a problem anymore. Recently ion-exchange albumin-heparin conjugate microspheres (AHCMS) were developed, which were shown to be biodegradable and biocompatible and which could be loaded with ADR up to payloads of 34% [10–13]. The AHCMS are prepared from a soluble conjugate of albumin and heparin. Pendent amino groups of the lysine residues of the albumin are used to crosslink the material in order to prepare stable microspheres. The anionic groups of the heparin (such as sulfamate groups and carboxylic acid groups) allow ionic interaction with the ADR, thus enabling high payloads and ion-controlled drug release.

Here we describe the preparation of novel synthetic biodegradable ion-exchange microspheres, based on polylysine. This polymer was partially sulfamated in order to allow crosslinking and to obtain the appropriate ion-exchange properties. The synthesis of the polyly-

sine and sulfamated polylysine and the testing of the cytotoxicity of the materials are described. The preparation of microspheres from partially sulfamated polylysine (SPLMS) and their characterization with regard to the swelling behavior are reported. Furthermore, the ADR loading and in vitro release characteristics of the SPLMS are reported.

2. Materials and methods

2.1. Materials

Benzylchloroformate, bis(trichloromethyl) carbonate (triphosgene), and hexanes were purchased from Janssen Chimica, Belgium. Anhydrous hydrogen chloride and anhydrous hydrogen bromide were purchased from Hoekloos, The Netherlands. Chlorosulfonic acid was purchased from Fluka, Belgium. L-Lysine·HCl was purchased from Merck, Darmstadt, Germany. Dry tetrahydrofuran (THF) and dioxane (Merck) were obtained by distillation from metallic sodium. Pyridine (Merck) was distilled in the presence of tosylchloride prior to use. Dextran T10 with a M_w of 10 000 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Highly refined olive oil was obtained from Sigma Chemical Company, St. Louis, MO, USA. Glutaraldehyde (25% aqueous solution), obtained from Merck, was purified by distillation [14] and adjusted to 2.5% and 5% w/v aqueous solutions prior to use. Phosphate buffered saline (PBS) was obtained from NPBI, Emmer Compascuum, The Netherlands. ADR was a kind gift from Pharmachemie, Haarlem, The Netherlands. All other reagents were obtained, in the highest possible grade, from Merck.

Thin layer chromatography (TLC) was performed with Merck precoated Silica 60 F254 plastic sheets (thickness 0.2 mm). Spots were visualized by spraying with ninhydrin solution. IR spectra were recorded using a Perkin-Elmer 1310 infrared spectrophotometer. NMR spectra were recorded using a Bruker AC 250 F spectrometer. Elemental analyses were carried out by the Laboratory of Chemical Analysis of the University of Twente.

2.2. Synthesis of poly-L-lysine

Poly-L-lysine (PL, Fig. 1,1) was prepared according to procedures previously described, using a triethy-

Fig. 1. Sulfamation of polylysine (1) using the pyridinium-sulfate complex (PSC, 2), yielding partially sulfamated polylysine (SPL, 3); $R = H, SO_3^-$. The degree of sulfamation is defined as the percentage of sulfamated lysine residues in the polymer.

lysine initiated ring-opening polymerization of the appropriate N^ϵ -carboxyanhydride [15,16]. First N^ϵ -benzyloxycarbonyl-L-lysine (Z-L-lysine) was prepared, followed by conversion of the ϵ -amino group-protected monomer to the NCA using triphosgene. Ring-opening polymerization, using triethylamine as an initiator, yielded the poly-Z-L-lysine. Finally the polymer was deprotected to give PL·HCl.

Z-L-lysine was prepared as described by Erickson and Merrifield [17]: upon formation of the copper-chelate complex of L-lysine by complexation of L-lysine with basic cupric carbonate to protect the α -amino group, the ϵ -amino group was protected using reaction with benzylchloroformate in the presence of NaOH. Subsequently, the copper was removed by repeated washing with ethylenediaminetetraacetic acid (EDTA). Next, Z-L-lysine-NCA was prepared as described by Daly and Poché [18]. In this procedure Z-L-lysine, suspended in THF, was reacted with triphosgene, yielding Z-L-lysine-NCA. After repeated recrystallization steps to remove chloride impurities, the Z-L-lysine-NCA was polymerized as described by Fasman et al. [19], using triethylamine as an initiator. Subsequently HCl and HBr were led through the poly-Z-L-lysine solution, followed by extensive dialysis of the solution against 0.01 M HCl using cellulose Visking® membranes to give the PL·HCl.

To determine the molecular weight of the poly-Z-L-lysine intrinsic viscosities were measured in dimethylformamide, using an Ubbelohde capillary dilution viscometer (100 L20, Canon) at 25°C. The molecular weight (M_v) was calculated from the limiting viscosity number, $[\eta]$, using the equation given by Daniel and Katchalski [20]:

$$[\eta] = 2.24 \cdot 10^{-7} (M_v)^{1.26} \text{ [dl/g]} \quad (1)$$

Viscosity measurements of PL·HCl were done in an aqueous solution, using an Ubbelohde capillary dilu-

tion viscometer (100 L20, Canon) at 25°C. The degree of polymerization (DP) was calculated from the specific viscosity, using Eq. 2. Since this empirical relationship was determined for a 1% w/v solution of PL·HBr [21], the requisite concentration of PL·HCl solution was calculated to be 0.787% w/v.

$$\log DP = 0.79 \log \eta_{sp} (c = 1\%) + 2.46 \quad (2)$$

2.3. Sulfamation of poly-L-lysine

PL can be sulfamated in aqueous alkaline solution, using the pyridinium-sulfate complex (PSC, Fig. 1,2). This compound has been widely used as a reagent in the sulfamation of amines but also for sulfating alcohols, sterols and carbohydrates [22].

The PSC was synthesized according to Dorlars [23]. Pyridine (60 ml) and chloroform (60 ml) were mixed and cooled to 2°C. The solution was vigorously stirred and chlorosulfonic acid (20 ml) was slowly added in small drops over a period of 1 h. Immediately a precipitate was formed and the temperature was kept below 15°C. After stirring for another 30 min at 10°C, the white precipitate was filtered and extensively washed with chloroform.

PL was sulfamated according to the method of Warner and Coleman [24]. In a typical experiment PL·HCl (105 mg) was dissolved in distilled water (30 ml). The pH of the solution was adjusted to 9.5 using 1.0 N NaOH. The mixture was stirred thoroughly at room temperature, and the PSC was added in small portions. The pH of the reaction medium was maintained at 9.5 by the continuous addition of 1.0 N NaOH, using a pH Stat (614 Impulsomat, Metrohm) until a precipitate formed. Then the pH was raised to 11.0. After addition of the PSC, the reaction mixture was dialyzed twice against 0.2 M aqueous NaCl and twice against distilled water. Subsequently the sulfamated PL (SPL, see Fig. 1,3) was freeze-dried and stored at 4°C. The degree of sulfamation ($DS = Y/(X + Y) \times 100\%$, with Y: fraction $R = SO_3^-$ and X: fraction $R = H$) was calculated from the 1H -NMR spectrum in D_2O .

2.4. In vitro toxicity testing of sulfamated polylysines

Toxicity of soluble sulfamated polylysines was evaluated using a tetrazolium-based colorimetric assay [25]. In this test, viability of cells is assessed by the

metabolism of a water-soluble tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into insoluble formazan salts. Cytotoxicity was tested using two cell lines, B16F10 colon carcinoma and P388 murine leukemia, which grow in monolayer or in suspension, respectively. B16F10 cell suspensions (20 000 cells per ml medium) were prepared in EMEM (Earle's Minimum Essential Medium, ICN Flow, Rickmansworth, Herts., UK), containing 10% v/v fetal calf serum (Imperial Ltd., UK), supplemented with 1% w/v sodium pyruvate, 1% v/v non-essential amino acids, 1.5% v/v vitamins and 1% v/v L-glutamine. P388 cell suspensions were prepared in RPMI medium (ICN Flow), containing 10% v/v fetal calf serum (Imperial Ltd.), supplemented with 1% v/v L-glutamine.

Cells were seeded into a 96-well plate, using 100 μ l of cell suspension per well. Sulfamated polylysines (10 mg) were dissolved in 1 ml of the respective medium and serial dilutions were prepared. Subsequently sulfamated polylysine solutions (100 μ l) were added to each well to give final concentrations of 0.01, 0.1, 0.5, 1.0 and 5.0 mg/ml (B16F10: $n=4$, P388: $n=3$; controls: B16F10: $n=20$, P388: $n=5$). After 67 h incubation at 37°C, MTT was added and cells were incubated for 5 more hours at 37°C. After this time, suspensions were centrifuged at 2000 rpm and the medium was removed. The formazan crystals were dissolved in DMSO (200 μ l/well) and the absorbance was measured at 550 nm, using an automatic Titertek Multiscan Plus plate reader (ICN Flow).

2.5. Oxidation of dextran

In this reaction vicinal diols of dextran are converted into dialdehydes. Dextran was oxidized using sodium metaperiodate as described by Schacht et al. [26,27]. In a typical experiment dextran T10 (12.15 g) was dissolved in 100 ml of distilled water. A solution of 6.42 g sodium metaperiodate in 100 ml of distilled water was added and the mixture was stirred in the dark at room temperature for 3 h. KI (20 ml, 4.5 M) was added in order to convert iodate to iodine and the mixture was dialyzed extensively against distilled water at 4°C and freeze-dried.

The aldehyde content of the oxidized dextran (Dex_{ox}) was determined by the hydroxylamine method [27,28]. Hydroxylamine hydrochloride (5 ml, 1.0 M)

was added to a 20 ml aqueous solution of Dex_{ox} , containing approximately 2 mmol aldehydes, calculated from IO_4^- consumption. The resulting solution was stirred for 24 h at room temperature, to ensure that all aldehyde groups had reacted with hydroxylamine hydrochloride. The mixture was then titrated potentiometrically using 0.1 N sodium hydroxide solution. The Dex_{ox} used in the microsphere preparations had a degree of oxidation (DO) of 29%.

2.6. Preparation of sulfamated polylysine microspheres

SPL microspheres (SPLMS) were prepared from SPL with $\text{DS} = 80 \pm 5\%$ (SPL80). Glutaraldehyde stabilized microspheres were prepared according to methods previously described for the preparation of albumin-heparin conjugate microspheres [10]. To a flat-bottomed glass beaker (diameter 60 mm, height 110 mm) equipped with four baffles (4 mm depth) positioned against the wall of the beaker was added 125 ml of olive oil. A four-bladed axial-flow impeller, motor-driven using a IKA-RW20DZM mechanical stirrer (Janke & Kunkel, Staufen, Germany) was placed in the center of the beaker with a 3 mm distance between the baffles and the impeller blade. The impeller was submerged for two thirds into the oil. After pre-stirring for 30 min at 850 rpm, 100 mg of SPL80 dissolved in 0.6 ml PBS was added. After emulsification for 15 min at 950 rpm, 0.15 ml of a glutaraldehyde solution was added giving a final crosslinker concentration of 0.5 or 1.0% w/v, thus preparing SPLMS05 and SPLMS10, respectively. The emulsion was stirred at 950 rpm for 3 h whereupon 50 ml of acetone was added and the resulting emulsion was stirred for 10 more minutes. SPLMS05 (or SPLMS10) were isolated by centrifugation for 15 min at 2700 rpm, followed by decantation of the supernatant. The microspheres were washed several times with acetone and finally dried in vacuo at 50°C.

Dex_{ox} stabilized SPL microspheres (SPLMSd) were prepared using similar procedures. SPL80 (100 mg) was dissolved in 0.6 ml of PBS and emulsified in 125 ml pre-stirred (950 rpm, 30 min) olive oil. After 15 min, 0.15 ml of PBS containing 0.02 g of Dex_{ox} was added and the emulsion was stirred for 24 h. Then 100 μ l of a NaBH_3CN solution (5 M) was added to reduce

the imines, and the emulsion was stirred for another 3.5 h. SPLMSd were collected as described above.

Glutaraldehyde stabilized and Dex_{ox} stabilized PL microspheres (PLMS05 and PLMSd) were prepared by similar procedures as described for SPLMS. However, due to increased viscosity of the PL solutions (as compared with SPL solutions of the same concentration) a stirring speed of 1050 rpm was applied.

2.7. Swelling experiments

The equilibrium swelling of SPLMS and PLMS was studied as a function of pH and ionic strength. The effect of the pH was studied using citrate and phosphate buffers at an ionic strength of 0.4. The effect of the ionic strength on the swelling was studied in phosphate buffers at pH 7.4. The ionic strength was adjusted using NaCl. SPLMS were resuspended in 15 ml of the swelling medium. The swelling ratio, Q (Eq. 3), was determined by measuring the total volume of 90 mg of swollen microspheres, resuspended in the swelling medium in calibrated vials, after centrifugation at 2000 rpm for 10 min.

$$Q = \frac{V_{\text{swollen}}}{V_{\text{dried}}} \quad (3)$$

2.8. Adriamycin loading of SPLMS

In a typical experiment, 5 mg of SPLMS was ultrasonically suspended in 1 ml of an ADR solution (7.5 mg/ml). This suspension was gently rotated for 20 h at 4°C, in the dark. The suspension was then centrifuged, the loading solution was decanted and 1 ml of distilled water was added to rinse the microspheres. After centrifugation, the washing solution was decanted again. This procedure was repeated three more times and was subsequently repeated four times with 1 ml of acetone to dehydrate the microspheres. The microspheres were dried in vacuo for 15 h at 50°C.

The maximal payload of the microspheres was determined using ADR loading solutions of increasing concentration (2.5–12.5 mg/ml). The amount of ADR remaining in the loading solution and the ADR amounts in the washing solutions were determined by UV/Vis measurements at 494 nm with a Uvikon 930 spectrophotometer (Kontron Instruments, Rotkreuz, Switzerland). The payload was calculated from the ADR

depletion of the loading solution and the ADR recovered from the washing and dehydration solutions.

2.9. Adriamycin release from SPLMS

Release experiments were carried out using a batch system. Microspheres (± 2 mg) were weighed into a 20-ml polypropylene (PP) vial. Ethanol (200 μ l) was added and the microspheres were ultrasonically resuspended. At $t=0$, 20 ml of release medium (PBS or distilled water) was added and the vials were put into a shaking bath at 37°C. The amplitude of the shaking was 2.0 cm and the frequency approximately 2 Hz. At intervals, the PP vials were taken from the bath and shaken vigorously to homogenize the suspension. A 1-ml sample was pipetted from the vial into a 1.5-ml Eppendorf® cup. After centrifugation, the supernatant was transferred into polystyrene micro-cuvettes and the A_{494} was measured to determine the amount of released ADR.

3. Results and discussion

3.1. Synthesis of sulfamated polylysine

PL·HCl was synthesized from L-lysine using a multi-step procedure in an overall yield of 11.6%. As can be seen in Table 1, the low overall yield was mainly caused by the low yield of the Z-L-lysine-NCA preparation step. The low yield of this reaction step was due to repeated recrystallizations carried out to reduce the Cl[−] content.

The results of the elemental analysis (Table 1) and the ¹H-NMR spectrum (Fig. 2a) revealed that the obtained PL was highly pure. This was confirmed by TLC and by the IR spectrum (data not shown).

The molecular weight (M_n) of the poly-Z-L-lysine calculated from the limiting viscosity number using Eq. 1 was 395 000. This corresponds to a degree of polymerization of 1510. The degree of polymerization as determined from the specific viscosity of a 0.787% solution of poly-L-lysine·HCl in water and calculated using Eq. 2 was 1509, indicating that the deprotection of the poly-Z-L-lysine under strong acidic conditions did not result in degradation of the polymer.

Sulfamation of the PL was performed by stepwise addition of a pyridinium-sulfate complex (PSC) and

Table 1
Results from poly-L-lysine preparation procedure

Product	Yield ^a (%)	Elemental analysis ^b (%)			
		C	H	N	Cl
Z-L-lysine	71	60.3 (60.0)	7.3 (7.2)	9.9 (10.0)	
Z-L-Lysine-NCA	25	59.3 (58.8)	5.8 (6.3)	8.9 (9.1)	<0.03 ^c
Poly-Z-L-lysine	76	64.4 (64.1)	7.0 (6.9)	10.6 (10.7)	
Poly-L-lysine · HCl	86	43.3 (43.8)	8.1 (8.0)	16.7 (17.0)	20.4 (21.5)

^aYield of the separate reaction steps; overall yield is 11.6%.

^bCalculated percentages are given in parentheses.

^cCl[−] content as determined by potentiometric titration with AgNO₃.

carried out at pH 9.5. Upon addition of high amounts of PSC, a precipitate was formed at a PSC/NH₂ molar ratio of approximately 0.7. This precipitate could be dissolved by raising the pH to 11 and adding the remainder of the PSC. This precipitation is probably due to intra- and intermolecular ionic interaction between the sulfamate groups and the remaining amino groups, which is impeded at higher pH and higher degrees of sulfamation. After dialysis and freeze-drying of the products, sulfamated PLs (SPLs) were obtained in a yield of 95%.

The ¹H-NMR spectrum of 100% sulfamated PL is shown in Fig. 2b. The δ-CH₂ and the β-CH₂ protons of PL · HCl are located at δ = 1.6–1.9 (see Fig. 2a and Table 2). In SPL the δ-CH₂ protons of a sulfamated lysine residue shift upfield to δ = 1.47–1.65 (see Fig. 2b and Table 2). A change in the chemical shift of the δ-CH₂ protons, without a change in the chemical shift of the ε-CH₂ protons upon sulfamation of a lysine residue, is expected on the basis of literature data of aliphatic ammonium salts and alkyl sulfonamides. The chemical shifts of α-CH₂ and β-CH₂ protons of aliphatic ammonium salts (comparable with ε-CH₂ and δ-CH₂ protons of lysine · HCl) are 2.9–3.1 and 1.7–1.8, whereas the chemical shifts of these α-CH₂ and β-CH₂ protons of alkyl sulfonamides (comparable with ε-CH₂ and δ-CH₂ protons of sulfamated lysine) are 2.9–3.45 and 1.45–1.6, respectively [29].

If the PL is only partially sulfamated, the δ-CH₂ protons of the unsulfamated NH₂-residues will be located at δ = 1.65–1.9 while the δ-CH₂ protons of sulfamated residues will be found at δ = 1.47–1.65. This is confirmed by the results obtained from the 2D-¹H-NMR experiments of PL (Fig. 3a), SPL100 (Fig. 3b)

and partially sulfamated SPL (Fig. 3c). In the PL spectrum, the interaction between the ε-CH₂ protons at δ = 2.9–3.2 and the δ-CH₂ (and β-CH₂) protons at δ = 1.6–1.9 is clear. In the SPL100 spectrum, no interaction between the ε-CH₂ protons (δ = 2.9–3.2) and the β-CH₂ protons (δ = 1.65–1.9) is present. Only interaction between the ε-CH₂ protons and the δ-CH₂ protons of the sulfamated lysine residues (δ = 1.47–1.65) is seen. In the partially sulfamated PL, both interactions are evident, indicating that the δ-CH₂ protons absorb at different δ depending on the sulfamation of the ε-NH₂ group. Since the relative peak areas in a ¹H-NMR spectrum are proportional to the number of absorbing protons, the DS can be calculated from the peak areas of the δ-CH₂ and β-CH₂-protons using Eq. 4.

$$DS = \frac{A_{1.55}}{A_{1.55} + A_{1.7}} \times 2 \times 100\% \quad (4)$$

with A_{1.55} being the δ-CH₂ peak area of a sulfamated lysine residue, absorbing at δ = 1.48–1.65 and A_{1.7} being the δ-CH₂ (of the unsulfamated lysine residues) + β-CH₂ peak area absorbing at δ = 1.65–1.9. The DS, calculated from the ¹H-NMR spectra of several SPLs, is depicted in Fig. 4. This graph shows that the DS can be controlled by the initial molar ratio of PSC/PL-ε-NH₂ groups.

3.2. In vitro cytotoxicity of sulfamated polylysines

Polylysine is known to be toxic both in vitro [25,30] and in vivo [31,32]. Cytotoxicity of the polylysine is probably due to the electrostatic interactions of the positively charged polymer with one or more essential,

Fig. 2. ^1H -NMR spectra of (sulfamated) poly-L-lysines in D_2O . (a) Poly-L-lysine $\cdot \text{HCl}$; (b) 100% sulfamated poly-L-lysine. $\delta = 5.6\text{--}5.9$: H_2O resonance, other peak assignments are given in Table 2.

Table 2
Assignment of PL and SPL100 ^1H -NMR resonances

Group	Chemical shift region (ppm) ^a	
	PL	SPL100
$\alpha\text{-CH}$	4.2–4.4	4.2–4.4
$\beta\text{-CH}_2$	1.6–1.9	1.65–1.9
$\gamma\text{-CH}_2$	1.3–1.6	1.3–1.47
$\delta\text{-CH}_2$	1.6–1.9 (1.7–1.8)	1.47–1.65 (1.45–1.6)
$\epsilon\text{-CH}_2$	2.9–3.2 (2.9–3.1)	2.9–3.2 (2.9–3.45)

^aLiterature values of $\alpha\text{-CH}_2$ and $\beta\text{-CH}_2$ resonances of aliphatic ammonium salts and alkyl sulfonamides are given in parentheses [29].

mostly negatively charged, cell components or structures, blocking or altering their function [30,31]. Molecular weight of the polymer also plays an important role in this process. Arnold et al. [32] demon-

strated that poly-L-lysine toxicity increased with increasing molecular weight.

In vitro cytotoxicity experiments showed that the toxicity of the polylysines was reduced significantly if the amino groups were sulfamated. Fig. 5 shows the relative viability of B16F10 cells after incubation with PL, SPL100 or SPL80. The sulfamated polylysines were tolerated at a concentration of approximately 1 mg/ml, whereas the polylysine was already toxic at the lowest tested dose (0.01 mg/ml).

The same trend was observed with the P388 MTT assay (Fig. 6), sulfamated polylysines (with DS ranging from 80% to 100%) were not toxic up to a dose of 5 mg/ml, whereas the unsulfamated PL was already toxic at the lowest tested dose (0.01 mg/ml). The large spread of the P388 data was due to plate variation. These results show that the toxicity of the PL was reduced significantly if the polymer was sulfamated at

Fig. 3. Contour plots of 2D-¹H-NMR spectra in D₂O from $\delta = 3.2$ to $\delta = 1.2$. (a) Poly-L-lysine · HCl; (b) 100% sulfamated poly-L-lysine; (c) partially sulfamated poly-L-lysine (SPL80).

Fig. 4. Degree of sulfamation of polylysine after reaction with pyridine-sulfate complex as a function of the initial PSC/PL-NH₂ ratio, as calculated from ¹H-NMR spectra of the resulting SPLs.

Fig. 5. Relative viability of B16F10 cells after incubation with (sulfamated) polylysines, as determined using a MTT assay. ●: PL; ○: SPL80; □: SPL100. Error bars indicate SD.

Fig. 6. Relative viability of P388 cells after incubation with (sulfamated) polylysines, as determined using a MTT assay. ●: PL; ○: SPL80; □: SPL95; △: SPL100. Error bars indicate SD.

Table 3
Results from SPL80 and PL microsphere preparation

Type	Crosslinking	Yield after preparation ^a (%)	10–40 μ m fraction (%)
SPLMS05	0.5% glutaraldehyde	73	65
SPLMS10	1.0% glutaraldehyde	71	72
SPLMSd	oxidized dextran	70	39
PLMS05	0.5% glutaraldehyde	75	61
PLMSd	oxidized dextran	68	42

^aBased on the initial amount of SPL or PL.

a DS of 80% and higher. There were no significant differences in cytotoxicity between the SPLs with a DS ranging from 80 to 100%.

3.3. Preparation and adriamycin loading and release properties of sulfamated polylysine microspheres

Pilot experiments showed that gelation of SPL solutions by addition of glutaraldehyde depended on the DS. At low DS (<90%), gels were rapidly formed upon addition of glutaraldehyde. At a DS of 95%, the viscosity of the polymer solution increased, indicating some crosslinking, however, this was insufficient to cause gelation. At DS = 100%, no changes in viscosity were observed at all. At DS = 35% solubility problems were manifest. These were probably due to the intramolecular ionic interactions of the ϵ -amino groups and the sulfamate groups. From these results it is concluded that a DS in a range of 60–90% is optimal for the preparation of SPLMS. SPLs with a DS in this range

contain sufficient ϵ -amino groups for crosslinking, whereas the relatively high amount of sulfamate groups ensures solubility and ion-exchange properties of the polymer.

Based on the results from the cytotoxicity and gelation experiments SPL with a DS of 80% (SPL80) was chosen as matrix material for the SPLMS. Microspheres were prepared from SPL80 and PL by the emulsion stabilization method using glutaraldehyde or Dex_{ox} as crosslinking agents. The aldehyde group content of the latter crosslinking agent could be varied by changing the initial molar periodate/glucoside ratio during the oxidation procedure of the dextran. The Dex_{ox} used in the microsphere preparations was prepared at an initial periodate/glucoside ratio of 0.4. The oxidation reaction was complete within 30 min and resulted in a DO of 29%. GPC measurements which were carried out using dextran fractions with a known narrow molecular weight distribution, indicated that the dextran was not degraded during the oxidation procedure (data not shown).

The microspheres were prepared in a yield of approximately 70% (Table 3). Crosslinking with glutaraldehyde resulted mainly in the formation of spherical particles whereas in the crosslinking with Dex_{ox} also irregularly shaped, large particles were formed. Consequently, the fraction of microspheres between 10 and 40 μ m, which was obtained by sieving with 10- and 40- μ m sieves, was considerably smaller in the case of Dex_{ox} crosslinked microspheres, as compared with glutaraldehyde crosslinked spheres (Table 3).

The swelling ratios of the microspheres as a function of the pH are shown in Fig. 7. The swelling ratio of the SPLMS is independent of the pH, whereas the swelling ratio of PLMS decreased at pH > 7. This can be explained by the ionization of the pendent groups. Sulfamate groups are strong acidic groups with a pK of approximately 1. At pH 3 and higher, there will be no change in the ionization of these groups, and the degree of swelling does not change. In the case of PLMS, the pendent ϵ -amino groups have a pK of approximately 8. At high pHs, these groups are deprotonated and the repulsive forces in the network that are due to electrostatic interactions are decreased resulting in a decrease of the degree of swelling.

The influence of the ionic strength on the swelling behavior is shown in Fig. 8. This graph shows that the repulsion of the equally charged groups in the network

Fig. 7. Swelling ratio Q of SPLMS and PLMS as a function of the pH at ionic strength, $I=0.4$; □: SPLMS05; △: SPLMS10; ○: SPLMSd; ■: PLMS05; ●: PLMSd. Error bars indicate SD.

Fig. 8. Swelling ratio Q of SPLMS and PLMS as a function of the ionic strength at pH = 7.4; □: SPLMS05; △: SPLMS10; ○: SPLMSd; ■: PLMS05; ●: PLMSd. Error bars indicate SD.

is decreased by the counter ions in the medium, thus reducing the swelling ratio at higher ionic strength. In distilled water (ionic strength = 0) the swelling ratio is maximal and most profound in the case of SPLMS05, which swell up to a swelling ratio of 35 (data not shown).

Fig. 7 and Fig. 8 show that the swelling ratio Q (Eq. 3) of the SPLMS05 is higher than that of SPLMS10. This can be explained by increased retractive forces at increased crosslink densities, which prevent the hydrogel from expanding. Previously this was also observed with AHCMS of which the degree of swelling decreased with increased crosslink density [10,33].

Fig. 7 and Fig. 8 also show that SPLMS05 swell more than SPLMSd, whereas the opposite trend is observed with PLMS. A speculative explanation is the difference in glutaraldehyde crosslinks that may occur due to the high amount of ϵ -amino groups present in PL in comparison with the amount of ϵ -amino groups of SPL. The exact crosslinking mechanism of glutaraldehyde is not fully understood. It is generally accepted that the initial step is the formation of an imine by the reaction of an aldehyde group with a primary ϵ -amino group [34]. Subsequently, various reactions may occur, with the imine acting as an intermediate, the most important being the formation of pyridinium compounds by oxidative cyclization of the imine [35–37]. However, unsaturated α,β -condensation oligomers (with $n=1-40$) may also be formed due to reactions involving conjugated imines [34,36]. If many ϵ -amino groups are present like in the case of PL,

it is conceivable that more, shorter crosslinks are formed while in the case of SPL, which contains fewer ϵ -amino groups, extended crosslinks are formed. In the case of Dex_{ox} crosslinked microspheres, the length of the crosslinks is mainly determined by the number of reaction sites along the dextran molecule, which may be different for PL and SPL. Therefore, more research on the structure of both glutaraldehyde and Dex_{ox} crosslinks is necessary to provide an adequate explanation for the observed swelling behavior.

In comparison with AHCMS, the swelling ratio of the SPLMS and the PLMS is considerably higher. AHCMS were shown to have a maximal swelling ratio of 11 [10,33], whereas the maximal swelling ratio of SPLMS was almost 35. Chemoembolization therapy requires microspheres with a diameter of 10–50 μ m in the swollen state. In order to obtain SPLMS suitable for chemoembolization therapy smaller SPLMS (dry size) have to be prepared.

SPLMS and PLMS are hydrophilic and easy to resuspend in aqueous solutions. Since SPLMS also have anion-exchange properties, they could be loaded with ADR by incubating the microspheres in an ADR solution. This procedure allowed efficient drug loading and high ADR payloads of SPLMS. Fig. 9 shows that the maximum ADR payload of SPLMS was approximately 60%, and was reached at a ADR/MS weight ratio of 1.5. This indicates that the efficiency of the loading process was almost 100% up to this ratio. Fig. 9 also shows that there was influence from neither the degree of crosslinking nor the kind of crosslinker used on the

Fig. 9. Influence of the ADR/MS ratio on the payload after 24 h of drug loading. MS (5 mg) were resuspended in 1 ml of ADR solution (2.5–12.5 mg/ml). □: SPLMS05; △: SPLMS10; ○: SPLMSd; ■: PLMS05; ●: PLMSd.

Fig. 10. Cumulative ADR release from SPLMS in distilled water at 37°C. At $t = 90$ min PBS was added to increase the ionic strength of the release medium. $\square$: SPLMS05; $\triangle$: SPLMS10; $\circ$: SPLMSd.

loading capacity. Furthermore, Fig. 9 shows that PLMS, which have a net positive charge, could only be loaded with ADR up to a few percent.

Fig. 10 shows that the ADR release from SPLMS was controlled by the ionic strength of the medium. As the microspheres were suspended in distilled water at 37°C, no ADR was released at all. After 90 min, PBS was added to the suspension to give an ionic strength half that of PBS. Upon increasing the ionic strength of the medium, ADR was instantaneously released.

Fig. 11 shows that ADR SPLMS released 50% of the drug within 1 h after suspension in PBS. Fig. 10 and 11 also show that neither the degree of crosslinking nor the crosslinking agent influenced the release profile. The ionic strength in the experiment shown in Fig. 10 was half that of PBS. As can be observed from Fig. 10 and 11 this difference had no influence on the release,

Fig. 11. Cumulative ADR release from SPLMS in PBS at 37°C. $\square$: SPLMS05; $\triangle$: SPLMS10; $\circ$: SPLMSd.

in both cases approximately 80% of the ADR was released within 6 h. The remaining drug was not released within the next 18 h. It is possible that this drug is covalently bound to the matrix after reaction with aldehyde groups that may still be present after crosslinking. It is also possible, however, that the electrostatic bond of the drug with the matrix is stabilized by self-association of ADR. This phenomenon was also observed in soluble heparin-ADR complexes [38] and a possible cause of incomplete ADR release from AHCMS [11].

In comparison with AHCMS, the maximum ADR payload of SPLMS is much higher. AHCMS could be loaded up to a payload of 34% [11], whereas the SPLMS could be loaded up to a payload of 60%. AHCMS release approximately 30% of the ADR in non-ionic medium [11], while 0% of ADR was released from SPLMS. Both types of microspheres released 80–90% of the drug in physiological salt solution. These results suggest that the ion-exchange properties of SPLMS predominantly control the drug binding and release, whereas in the case of AHCMS, hydrophobic interactions also play an important role. Since SPLMS can in principle be modified by introduction of amino acids with uncharged or hydrophobic pendent groups in the polymer backbone, these microspheres can in principle be used as a model system to study the interaction of ADR with AHCMS. Whether SPLMS are suitable chemoembolization devices which could be used if high payloads are necessary depends on the *in vivo* behavior of these microspheres. This remains to be studied.

4. Conclusions

It was shown that polylysine can be sulfamated in a controlled way to obtain polymers ranging from 100% cationic to 100% anionic. Upon sulfamation, the *in vitro* cytotoxicity of polylysine was significantly reduced at degrees of sulfamation of 80% and higher. Sulfamated polylysines with a degree of sulfamation of 80% could be used to prepare microspheres (SPLMS) with a dry size of 10–40 μm in diameter, by techniques developed for the preparation of AMS and AHCMS. In comparison with AHCMS, the ADR loading and release characteristics of SPLMS were improved. The maximal payload was increased by 25% up to 60%, whereas release in a non-ionic medium was

completely absent. This indicates that a suspension of drug-loaded microspheres could be prepared in non-ionic medium like 5% glucose solution, without any drug release taking place. Upon intra-arterial administration of this suspension, drug release is triggered by ions in the blood.

These results show that sulfamated polylysines can be used to prepare ion-exchange microspheres which in turn have great potential as ADR delivery devices. In order to evaluate these microspheres for chemoembolization therapy, in vivo behavior of the sulfamated polylysine microspheres remains to be studied.

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