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Research paper

Protein adsorption patterns on poloxamer- and poloxamine-stabilized solid lipid nanoparticles (SLN)

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Abstract

Solid lipid nanoparticles (SLN) were produced using a full range of poloxamer polymers and poloxamine 908 for stabilization. The protein adsorption pattern acquired on the surface of these particles after intravenous injection is the key factor determining the organ distribution. Two-dimensional polyacrylamide gel electrophoresis (2-DE) was employed for determination of particle interactions with human plasma proteins. The objective of this study was to investigate changes in the plasma protein adsorption patterns in the course of variation of the polymers stabilizing the SLN. Considerable differences in the protein adsorption with regard to preferential adsorbed proteins were detected for the different stabilizers. Possible correlations between the polyethylene oxide (PEO) chain length and the adsorption of various proteins (first of all apolipoproteins) are shown and discussed. Besides the study of protein adsorption patterns, the total protein mass adsorbed to the SLN was also evaluated using the bicinchoninic acid (BCA)-protein assay. The knowledge concerning the interactions of proteins and nanoparticles can be used for a rational development of particulate drug carriers. Based on the findings presented in this paper, we anticipate that the in vivo well-tolerable SLN are a promising site-specific drug delivery system for intravenous injection.

Keywords: Solid lipid nanoparticles; Poloxamer; Poloxamine; Protein adsorption patterns; Two-dimensional polyacrylamide gel electrophoresis; Site-specific drug delivery

1. Introduction

Intravenously (i.v.) injected nanoparticulate drug carriers are one attempt to realize the 'magic bullet' concept postulated by Ehrlich, which means to target drugs specifically to their site of action [1]. To allow a controlled development of site-specific carriers, it is a prerequisite to know the factor determining the organ distribution of the carriers. Since more than half a century, intensive studies are being undertaken to identify this organ distribution determining factor [2–4]. Meanwhile, it is generally accepted that the protein adsorption pattern, acquired after intravenous injection of the particles, is the crucial factor determining the organ distribution [5,6]. Two-dimensional polyacrylamide gel electrophoresis (2-DE) has proven to be a powerful tool to determine these plasma protein adsorption patterns of nanoparticulate carriers [6]. In case opsonins such as immunoglobulin or complement factors are adsorbed, the particles are immediately cleared by the macrophages of the mononuclear phagocytic system (MPS) [7]. In case these opsonins are missing, the particles show a reduced or no uptake by MPS cells [8]. Such particles can circulate in the blood stream, e.g. poloxamine 908-coated 60 nm polystyrene model carriers [3,9]. An even more pronounced stealth effect could be achieved if the so-called dysopsonins (albumin or apolipoproteins) were preferentially adsorbed on the particles surface. In case of a pronounced albumin, adsorption occurs on the particle surface, the surface renders more hydrophilic reducing in general the adsorption of other blood proteins. To target a specific drug to a site other than the MPS, firstly the drug-loaded particles need to escape from the MPS recognition, and secondly, they need to have preferentially adsorbed on their surface a protein, which is able to mediate the uptake to the target cells. According to this

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theory, targeting to the brain was achieved using Tween 80-stabilized polymeric nanoparticles [10–12]. Apolipoprotein E (apoE) could be identified as the component mediating the brain targeting [13].

Most of the organ distribution, and related protein adsorption studies, were performed either with non-biodegradable model particles (e.g. polystyrene) or using polymeric nanoparticles. This latter system usually exhibits the problems such as too slow in vivo biodegradation and release of toxicologically problematic degradation products such as formaldehyde (e.g. polyalkylcyanoacrylate nanoparticles [14]). Therefore, in this study, solid lipid nanoparticles (SLN) were used. The SLN showed a very good tolerability in vitro cell culture studies [15] but also after intravenous bolus injection of up to 1 g lipid/kg [16,17]. Based on this, the SLN have the potential to be accepted by the regulatory authorities and being used in patients.

Efficient surface modifiers that can be used to reduce the MPS uptake are polyethylene oxide (PEO)-containing nonionic block co-polymers, i.e. poloxamers and poloxamines, also known as Pluronic® and Tetronic®, respectively. It was shown in vivo that particles coated with such polymers could circulate longer in the blood (poloxamine 908) [3] or be accumulated in the bone morrow (poloxamer 407) [18]. Moreover, studies on bovine brain endothelial cells have demonstrated that poloxamer 235 (Pluronic P85) single chains ('unimers') inhibited the glycoprotein P (P-gp) efflux pump, thus enhancing drug accumulation in these cells [19]. In contrast, poloxamer 235 micelles induced transient drug accumulation in the same cells. The efflux is directed to the same side where the micelles were administered. However, the direction of transport can be modified by conjugating the micelles with a ligand capable of adsorptive endocytosis in the cell [20], such as apoE.

Therefore, the SLN investigated in this study were surface-modified with a range of these interesting polymers to assess whether they show adsorption patterns known from model or polymeric particles leading to site-specific accumulation (targeting). In case such patterns are found, SLN could be used as a well-tolerated i.v. targeting delivery system.

2. Materials and methods

2.1. Materials

Cetyl palmitate was obtained from Henkel KG (Düsseldorf, Germany), poloxamer 184, 235, and 407 from C.H. Erbslöh (Düsseldorf, Germany), poloxamer 188 from BASF (Ludwigshafen, Germany) and poloxamer 237, 238, 338, and poloxamine 908 from ICI Surfactants (Middlesborough, UK). Immobiline DryStrips (pH 3–10, nonlinear) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden) and human plasma was obtained from the German Red Cross (Berlin, Germany) and stored at −70 °C. For 2-DE, all chemicals were of analytical grade. Acrylamide was purchased from Serva (Heidelberg, Germany) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate and piperazine diacrylamide (PDA) from BioRad (Munich, Germany). All other chemicals according to [21] were obtained either from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). The BCA regent-kit for protein quantification was obtained from Pierce (Rockford, USA).

2.2. Methods

2.2.1. Solid lipid nanoparticles: preparation and physicochemical characterization

SLN were produced by the hot homogenization method as described previously [22,23] using cetyl palmitate as matrix lipid (10.0% (w/w)). The polymer concentration (poloxamer, poloxamine) was kept constant at 1.2% (w/w). The lipid was melted at approximately 5 °C above its melting point and dispersed by an Ultra-Turrax T 25 (Janke and Kunkel, Staufen, Germany) in a hot surfactant mixture heated at the same temperature. The obtained pre-emulsion was then homogenized at the same temperature using a Micron LAB 40 (APV Systems, Unna, Germany), applying three homogenization circles at 500 bar. A hot nanoemulsion resulted; cooling led to crystallization of the lipid and formation of the SLN.

The mean diameter of the nanoparticle population was assessed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer IV (Malvern instruments, Malvern, UK). PCS gives the mean diameter of the particle population and the polydispersity index (PI) ranging from 0 (monodisperse) to 0.50 (very broad distribution). The content of micrometer particles was determined by laser diffractometry (LD) using a Coulter LS 230 (Beckman-Coulter, Krefeld, Germany). LD yields a volume distribution. The diameters 50, 90, 95 and 99% were chosen as parameters characterizing the size distribution (i.e. a diameter of 95% means that 95% of the particle volume is below the given size).

Zeta potential measurements were performed using the Malvern Zetasizer IV. The field strength applied was 20 V/cm. The SLN were dispersed in distilled water having a conductivity adjusted to 50 μS/cm by addition of NaCl-solution, pH was about 5.8. The conversion of the electrophoretic mobility to the zeta potential was done using the Helmholtz–Smoluchowsky equation.

Water contact angle on a cetyl palmitate film was measured using a Krüss G1 contact angle goniometer (Krüss, Hamburg, Germany) equipped with a sessile dropper. Care was taken to perform measurements on part of the film on which there were no cracks. Ten readings were taken and the mean value was obtained.

2.2.2. Sample preparation

To analyze the protein adsorption on the SLN, suspensions of the particles containing constant surface areas

(1.3 m^2, calculated on the basis of the measured PCS diameter) were incubated in 1.5 ml citrate stabilized human plasma for 5 min at 37 °C. The particles were separated from excess plasma by centrifugation, using a Biofuge 22R (Heraeus Sepatech, Hanau, Germany) at 22,940*g* for 60 min. Afterwards the particle pellet was resuspended in 1.5 ml 20 mM sodium phosphate buffer, pH 7.4 by vortexing and centrifugating again using the same conditions. This washing cycle was repeated three times. Removal of adsorbed proteins from the particle surface was performed according to Cook and Retzinger [24]. The pellet was mixed with 10 μl of a solubilizing solution containing 10% w/v sodium dodecyl sulfate (SDS) and 2.32% w/v dithioerythritol (DTE) and incubated for 5 min at 95 °C. After cooling, 190 μl of a solution containing DTE, cholamidopropyldimethylhydroxypropanesulfonate (CHAPS), urea, Tris, and bromophenol blue were added and the mixture was vortexed and centrifuged 15 min at 22,940*g*. One hundred microlitres of this solution were applied to first dimension of the 2-DE.

2.2.3. Two-dimensional polyacrylamide gel electrophoresis (2-DE)

2-DE was carried out as described by Blunk et al. [6] with equipment from Amersham Pharmacia Biotech (Uppsala, Sweden), Consort (Turnhout, Belgium) and BioRad (Munich, Germany). Within the first dimension (isoelectric focussing, IEF) using Immobiline DryStrips (pH 3–10, non-linear) from Amersham Pharmacia Biotech, the proteins are separated according to their isoelectric points (p*I*) [25]. In the second dimension (SDS-PAGE), separation of proteins takes place due to their molecular weight (MW) in 16 cm × 16 cm gel slabs, having a gradient from 9% T (total acrylamide concentration) to 16% T using 0.8% w/v PDA as cross-linker. Afterwards, the gels were silver stained according to Hochstrasser et al. [21] and scanned with an ImageScanner from Amersham Pharmacia Biotech. Since each protein spot has its own characteristic coordinates (p*I* and MW), identification can be performed by comparison of the obtained 2-DE gels with reference maps [26]. The spot intensity was evaluated using the software MELANIE III from BioRad. For data assessment, it has to be taken into account that the silver-staining density is a characteristic of each protein [27]. A quantitative comparison between identical spots is valid on different gels, but the same does not happen between different spots on the identical gel. Therefore, the data can be regarded as only semi-quantitative. Nevertheless, they can be used for a reliable approximation of the amount of proteins adsorbed.

2.2.4. Protein quantification

For the determination of the total protein amounts in different samples, the bicinchonic acid (BCA)-protein assay was used. This assay is suitable to measure even proteins covalently bound to surfaces [28].

The samples were prepared according to sample preparation prior to 2-DE (see Section 2.2.3). After the last washing step, the standard BCA-assay was performed as described in the manufacturer's instruction. Briefly, the particle pellets were mixed with 2 ml BCA working-reagent (50 parts reagent A containing BCA detection reagent in 0.1 N sodium hydroxide to 1 part reagent B containing copper sulfate). After incubation for 30 min at 60 °C, the samples were centrifuged in order to remove the particles (22,940*g*, 15 min, 7 °C to slow down the color reaction). The adsorption of the samples and a range of bovine serum albumin (BSA)-standard solutions were measured at 562 nm using an UV–VIS spectrometer (Uvikon 940, Kontron instruments, Eching, Germany).

3. Results and discussion

3.1. Size and charge of SLN

Table 1 shows the size and zeta potential characterization data of the particles. The mean PCS diameters of all particles were approximately in the range 220–280 nm, in addition, the laser diffractometry data were also relatively similar. Based on this, a size effect of the adsorption could be excluded or could only be responsible for a minor effect. In case of very large differences in the curvature of the surface, the packaging of the PEO tails and loops protruding into the water phase might show distinct differences (different exposure of more hydrophobic polypropylene (PPO) parts) potentially leading to differences in the adsorption patterns not related to the type of the stabilizer

Table 1
Size characterization data of cetyl palmitate SLN (10%) stabilized with various poloxamers (P-184 to P-338) and poloxamine 908 (P-908) by PCS (PI = polydispersity index) and by laser diffractometry (diameters 50–99%) as well as the zeta potential (mV)

System	1.2% P-184	1.2% P-235	1.2% P-237	1.2% P-188	1.2% P-238	1.2% P-407	1.2% P-338	1.2% P-908
PCS (nm)	219 ± 4	237 ± 3	263 ± 4	259 ± 5	264 ± 4	256 ± 4	286 ± 4	255 ± 4
PI	0.082	0.072	0.101	0.078	0.108	0.131	0.167	0.157
D50% (μm)	0.199	0.280	0.327	0.327	0.350	0.323	0.331	0.332
D90% (μm)	0.386	0.458	0.509	0.522	0.548	0.529	0.531	0.533
D95% (μm)	0.435	0.512	0.563	0.581	0.611	0.590	0.590	0.591
D99% (μm)	0.538	0.613	0.660	0.694	0.719	0.707	0.703	0.702
mV	−38.9	−28.8	−21.5	−23.8	−23.6	−19.4	−15.9	−11.9

Table 2
Characterization data of the different poloxamers (P-184 to P-338) and poloxamine 908 (P-908)

	P-184	P-235	P-237	P-188	P-238	P-407	P-338	P-908
Molecular weight	2900	4600	7700	8350	10800	11500	14000	25000
n	13	27	62	75	97	98	128	121
m	30	39	35	30	39	67	54	16
n/*m*	0.43	0.69	1.77	2.5	2.49	1.46	2.37	7.56
Coating layer thickness (nm)	2.4	3.5	8.0	7.6	13.2	11.9	15.4	14.4

$(PEO)_n-(PPO)_m-(PEO)_n$; PEO, polyethylene oxide unit; PPO, polypropylene oxide unit) and coating layer thickness of 60 nm coated polystyrene model particles (from [4]).

but to the degree of curvature and polymer conformation on the surface.

The zeta potential was the highest for the poloxamer of lower MW (poloxamer 184) (−38.9 mV) and decreased with increasing MW, being the lowest for poloxamine 908 (−11.9 mV). These results were expected once they are in agreement with the theory [4]. In fact, with increasing MW, the polymer adsorption layer thickness also increases, thus shifting the shear of plane to a further distance to the particle surface resulting in a decrease of the measured zeta potential [4]. The zeta potential (charge) cannot be kept similar because it depends on the properties of each polymer (i.e. MW, polymer adsorption layer thickness). According to this explanation, the analyzed protein adsorption patterns are likely to be affected by both the type of polymer and the charge being present. The charge effect was nicely demonstrated in adsorption studies using polymeric nanoparticles of similar size and similar surface hydrophobicity but possessing different zeta potentials [29]. Major differences were found when comparing positively charged and negatively charged polymeric particles. Positively charged particles adsorbed preferentially proteins with isoelectric point (p*I*) < 5.5 (e.g. haptoglobin β chain, albumin or α1-antitrypsin) and the negatively ones preferentially proteins with isoelectric point (p*I*) > 5.5 (e.g. immunoglobulin G or apoH) [30,31]. At least, in this study, all zeta potentials were negative.

3.2. Poloxamer/poloxamine adsorption onto surfaces

Table 2 lists the characterization data of the different polymers. The conformation of the adsorbed polymer is crucial for the sterically stabilizing effect, i.e. the physical stability of the suspensions. In addition, the conformation determines the protein adsorption pattern, i.e. via the degree of exposure of hydrophilic PEO chains or relatively hydrophobic PPO chains and steric hindrance of flexible polymer chains against the adsorption of proteins.

At very low polymer concentrations, the polymers adsorb in a very flat conformation on the surface. With increasing concentration of the polymer, the adsorbed polymer molecules are getting compressed leading to the formation of loops and tails (Fig. 1) [32]. Further, increase of the polymer concentration in the solution leads to a very dense packing of the loops affecting, therefore, the protein adsorption behaviur.

Studies have been performed regarding the reduction of total protein adsorption as a function of the length of PEO tails and the density on the surface [33–35]. These studied PEO chains were covalently linked to the surface of polymeric model particles. It could be shown that plasma protein adsorption strongly depends on the PEO chain length on the surface, as well as on the PEO chain density. A maximal

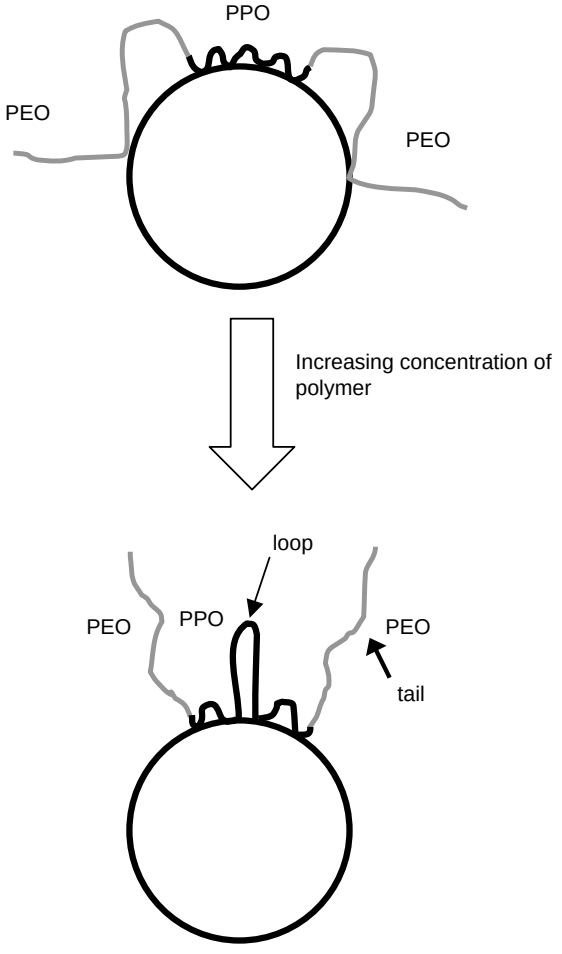

Fig. 1. Conformation of poloxamers in dependence on the polymers concentration.

reduction in protein adsorption was found for a PEO MW of 5000 [33]. Independently on the thickness and density of the corona, the qualitative composition of the plasma protein patterns was very similar, showing that the adsorption was determined by interaction with the surface of the core protected more or less by PEO chains. Gessner et al. [35] studied the effect of high surface densities (0.39–0.31 nm) achieved with very short PEO-chains (MW 200 and 400, respectively). Despite the increase of PEO-surface density, the total protein amount showed no considerable decrease of the protein adsorption, both for PEO-200 and for PEO-400. Stronger adsorption of IgG on all PEO-400 grafted particles, and the fact that several proteins (albumin, fibrinogen) show constant adsorption on all types of PEO-grafted particles, lead to the assumption that in this case the PEO-segments are involved in the adsorption process. This is in agreement with the findings of Carignano and Szleifer [34], which say that a very high surface coverage can modify the surface chemistry and may result in a protein attractive surface.

The polymer concentrations used to stabilize the SLN particles of the study were relatively high, which means that a compact loop-tail conformation can be assumed. When using polymeric model particles, the thickness of the adsorption layer can be easily determined by PCS and consequently conclusions can be drawn regarding the conformation [36]. The difference between the radii of coated and uncoated particles is equivalent to the coating layer thickness (hydrodynamic radius). The coating layers of the poloxamers and poloxamine 908 used in this study were previously determined on polystyrene model particles with sizes around 60 nm (Table 2).

The problem associated with SLN is the fact that a coating layer thickness cannot be determined by PCS. There are no stabilizer-free SLN available (they would immediately aggregate after production), which means that a thickness determination by calculating the difference between non-polymer stabilized and polymer-stabilized particles is not possible. To be more precise, it would be only possible when applying for example small-angle neutron scattering (SANS) measurements, which were not available to us. But, it is possible to assume a similar relationship of the thickness of the polymers to each other. That means poloxamer 338 and poloxamine 908 will provide the thickest layers. Due to their relative hydrophilic character, the block co-polymers will not protrude into the lipid matrices, they adsorb onto the SLN surface. One of the factors affecting the adsorption layer thickness is the surface hydrophobicity. One approach to quantify hydrophobicity of surfaces is measuring the contact angle [37], of course, having in mind the limitations of the method and also the fact that the two systems are difficult to compare once they do not have the same sizes (60 vs 240 nm).

The contact angle for polystyrene was 89.7° [38], the contact angle measured on a cetyl palmitate film was 87.8° (standard deviation of 2.5°). From this, the assumption of similar thickness as observed on the polystyrene model particles is supported (Table 2).

However, the most important and final organ-determining factor is the composition of the protein adsorption pattern, which was determined in this study.

3.3. Protein adsorption

Man prefers to have simple relationships—despite the fact that a rather complex biological process is investigated. Therefore, in the middle of the fifties one tried to relate one physicochemical parameter (e.g. the zeta potential) to the organ distribution of intravenously injected carriers [2,4]. Based on this, publications describing for example the uptake of particulate carriers by macrophages in cell culture related the uptake rate to the molecular structure of the particle-stabilizing polymers, e.g. length of PEO chains, length of PPO chains [39–41]. Of course, it should be clear that in such established relationships, the plotted parameter such as PEO chain length might not be directly related to an observed phenomenon. For example, the PEO chain length affects the conformation of the adsorbed polymer; consequently this conformation affects the protein adsorption.

Fig. 2 shows the plasma protein adsorption patterns of SLN stabilized with poloxamer polymers with MW lower than 10,000. Fig. 3 shows the patterns obtained from the SLN stabilized with polymers with MW higher than 10,000. Table 3 lists the volume percentages of the most abundant proteins on the different particles. From one preparation to another, the total amount obtained may vary as a function of variation in the particle number obtained from the separation process. Therefore, the proteins are expressed in the arbitrary unit percentage of volume. The percentage is calculated based on the total amount of protein adsorbed. This means that in case the relative percentage of the protein is high, but the total amount of adsorbed protein is low, the total amount present on the particle will also be low (despite the high relative percentage). However, not only the percentage is important. The total amount is also important because a minimum amount of protein present on the surface is considered to be necessary to mediate the adherence to a target cell.

Adsorption of apoE on nanoparticulate drug carriers is very interesting with regard to utilizing such carriers for the delivery of drugs to the brain. Successful targeting to the brain was previously shown for drug nanocrystals [42] and for polybutylcyanoacrylate nanoparticles stabilized with Tween 80 [43], both showing apoE adsorbed on the surface. Therefore, it was an interesting result that apoE was preferentially adsorbed when the SLN were stabilized with poloxamer having a low number of PEO units (Fig. 4). There is a nice exponential relationship between adsorbed apoE and the PEO chain length, showing highest adsorption of apoE when using poloxamer 184 and poloxamer 235. These results are in agreement with

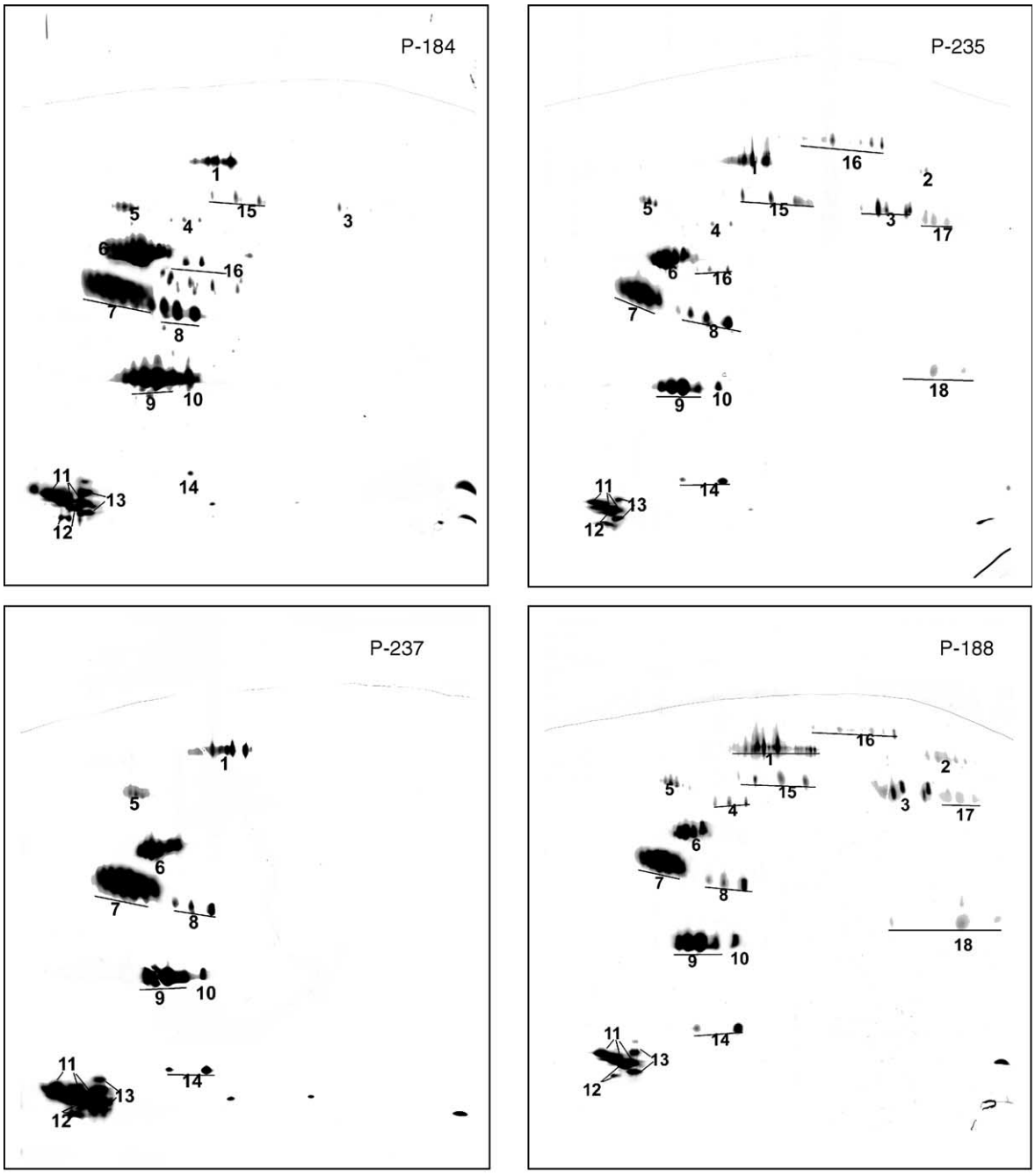

Fig. 2. 2-DE gels of SLN stabilized with poloxamer polymers with MW lower than 10,000. Upper left, poloxamer 184-stabilized SLN (P-184 SLN); upper right, P-235 SLN; lower left, P-237 SLN; lower right, P-188 SLN. The entire gels are shown, p*I* 4.0–9.0 (from left to right, non-linear), MW 250-6 kDa (top to bottom, non-linear). (1) Albumin, (2) fibrinogen α chain, (3) fibrinogen β chain, (4) fibrinogen γ chain, (5) α1-antitrypsin, (6) apoA-IV, (7) apoJ, (8) apoE, (9) apoA-I, (10) proapoA-I, (11) apoC-III, (12) apoC-II, (13) apoA-II, (14) transthyretin, (15) apoH, (16) IgM μ chain, (17) IgG γ chain, (18) Ig light chain.

the apoE values obtained by Blunk [44] with O/W emulsions (20% (w/w) soja oil) stabilized with the different poloxamers (2.5% (w/w)) used in this study (Fig. 4). Again, highest apoE adsorption occurred, when using polymers with short PEO blocks in the molecule (poloxamer 184 and poloxamer 235). The SLN stabilized with polymers with longer PEO chains (especially poloxamine 908, poloxamer 338 and poloxamer 407) showed a pronounced adsorption of fibrinogen. One should note that adsorption of additional protein would reduce the percent fraction of the other proteins. The reason for this additional adsorption remains unclear. However, when looking at the 2-DE gels, it is obvious that also the total amount of adsorbed apoE was very low or even no apoE was detected.

Weisgraber et al. [45] showed that apoC-I and apoC-II (but not apoC-III) inhibit the binding of apoE-containing

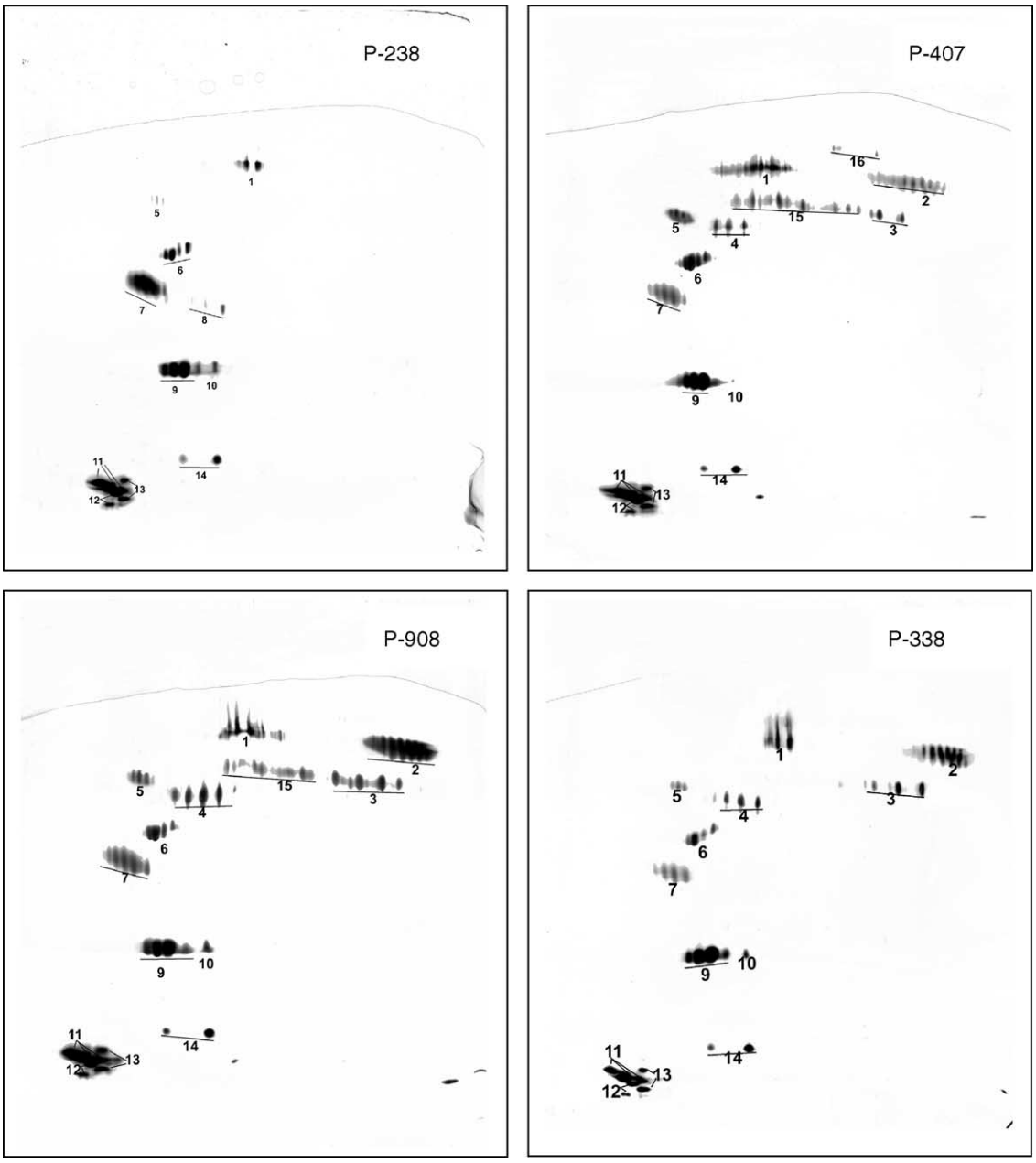

Fig. 3. 2-DE gels of SLN stabilized with polymers with MW higher than 10,000. Upper left, poloxamer 238-stabilized SLN (P-238 SLN); upper right, P-407 SLN; lower left, poloxamine 908-stabilized SLN; lower right, P-338 SLN. The entire gels are shown, p*I* 4.0–9.0 (from left to right, non-linear), MW 250-6 kDa (top to bottom, non-linear). (1) Albumin, (2) fibrinogen α chain, (3) fibrinogen β chain, (4) fibrinogen γ chain, (5) α1-antitrypsin, (6) apoA-IV, (7) apoJ, (8) apoE, (9) apoA-I, (10) proapoA-I, (11) apoC-III, (12) apoC-II, (13) apoA-II, (14) transthyretin, (15) apoH, (16) IgM μ chain.

lipoproteins such as β-very low density lipoproteins (β-VLDL) to the LDL receptor. The receptor mediated binding and uptake of lipoproteins (respectively particles) is enhanced by apoE and is inhibited by apoC-I and apoC-II. Therefore, to achieve targeting to the brain, it would be beneficial to have a high apoE/apoC-II ratio adsorbed on the nanoparticles. Fig. 5 shows the relationship between the apoE/apoC-II ratio and PEO chain length. Again the highest ratio is observed with SLN stabilized with poloxamer having a low number of PEO units, i.e. poloxamer 235 and poloxamer184.

Looking at the complete adsorption patterns, even when using poloxamers of low MW, typical opsonins such as immunogloblin or complement factors were not detected or were detected in a very low amount. However, it is well known that model polymeric nanoparticles stabilized with polymers of lower MW such as poloxamer 188 are not as efficiently protected against MPS uptake compared to particles stabilized with polymers of higher MW (e.g. poloxamer 407 or poloxamer 338 [9,46]). Therefore, these SLN might have the potential to deliver drugs to the brain, however, with a certain loss of particles to the MPS cells.

Table 3
Protein adsorption on poloxamer (P-184 to P-338) and poloxamine 908 (P-908)-stabilized SLN. Amounts of single proteins are expressed as percentages of the overall protein amount on the particles

	P-184	P-235	P-237	P-188	P-238	P-407	P-908	P-338
PEO-units (n)	13	27	62	75	97	98	121	128
Albumin	4.1±1.4	7.0±0.8	5.1±0.9	5.8±0.7	9.5±4.4	9.1±0.8	8.1±2.1	7.8±1.5
ApoE	10.4±0.5	6.3±0.5	2.6±0.4	2.3±0.3	1.3±0.2	0.5±0.5	0.3±0.3	0.1±0.1
ApoC-II	6.0±0.5	2.5±0.5	7.9±0.6	6.0±0.4	6.8±0.5	4.6±1.6	3.8±0.3	6.7±2.9
ApoA-IV	22.3±2.3	19.4±1.4	12.4±0.8	11.2±0.9	9.6±0.7	12.4±0.2	6.7±1.2	9.5±3.0
ApoJ	16.6±1.4	22.2±1.8	27.7±4.0	21.7±3.8	25.4±1.8	15.9±4.8	10.7±1.1	14.6±4.5
ApoA-I	17.7±0.7	15.1±0.2	14.5±1.4	19.0±1.2	18.7±2.2	19.0±0.9	11.2±1.7	20.0±2.0
ApoC-III	9.5±4.1	6.8±1.9	12.8±0.9	13.5±4.6	13.8±0.3	13.8±1.7	7.7±1.3	11.6±2.1
ApoA-II	3.6±0.6	2.9±0.5	7.6±0.8	6.0±1.8	6.4±0.9	5.1±0.6	3.3±0.6	5.4±1.0
ApoH	1.2±0.7	5.1±1.1	0.0±0.0	2.8±0.4	0.0±0.0	3.1±3.0	0.0±0.0	3.9±0.1
Fibrinogen	0.4±0.5	2.9±1.2	1.9±1.9	2.9±1.8	0.5±0.5	9.1±8.5	35.4±6.9	16.2±9.2

All the proteins with at least one measurement above 5% are listed (percentages are the means of three experiments, ± are the standard deviations).

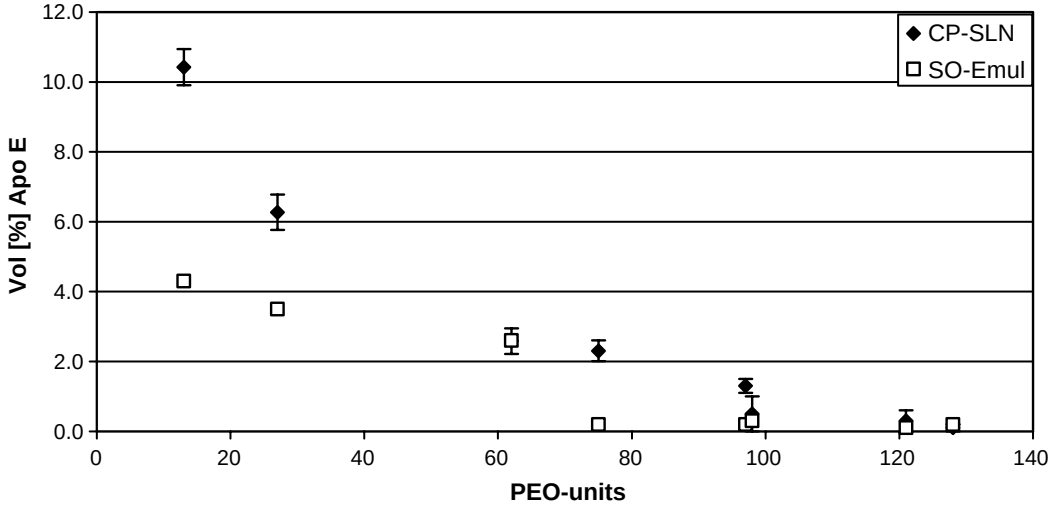

Fig. 4. Percentages adsorbed apoE on the cetyl palmitate SLN (CP-SLN) and soja oil O/W-emulsions (SO-Emul), respectively, in dependence on polyethylene oxide chain length of the used surfactants. Error bars represent the standard deviation ($n=3$), no error bars means $n=1$.

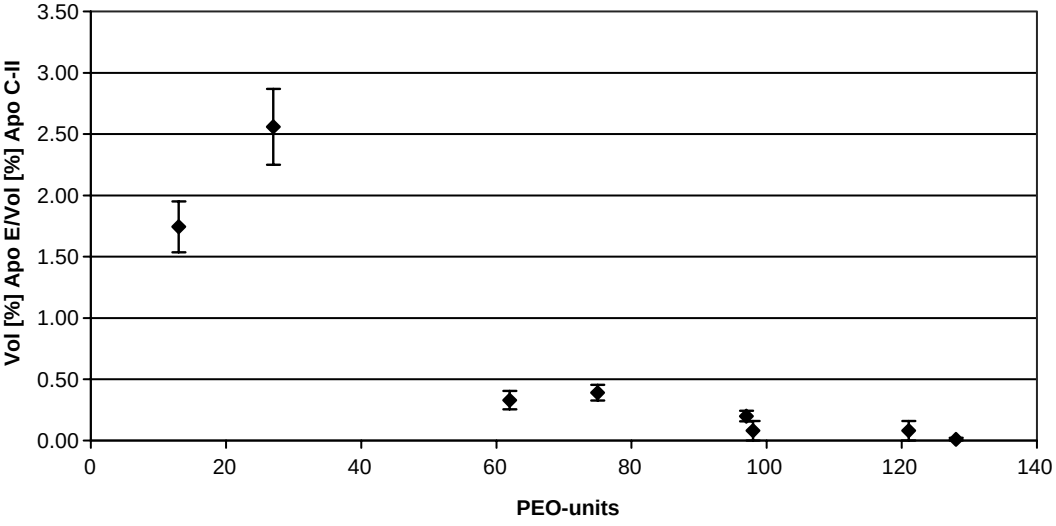

Fig. 5. ApoE/apoC-II ratio adsorbed on the SLN in dependence on polyethylene oxide chain length of the used surfactants. Error bars represent the standard deviation ($n=3$).

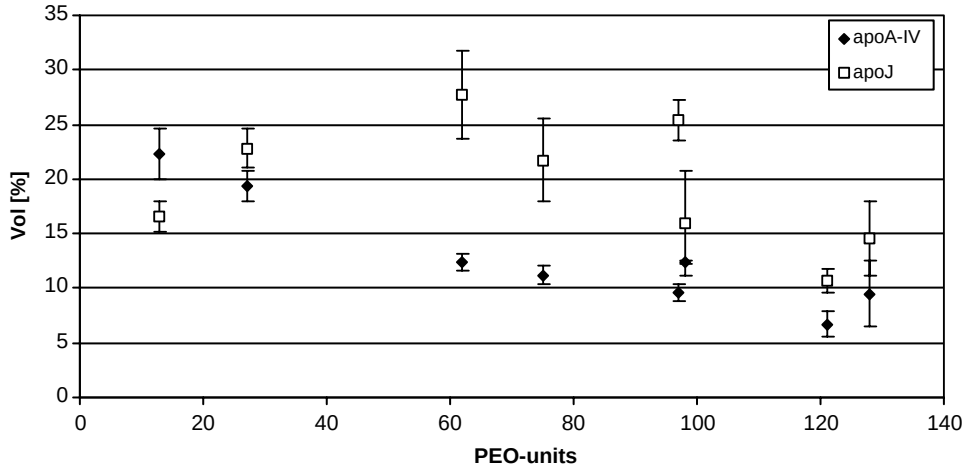

Fig. 6. Percentages adsorbed apoA-IV and apoJ on the cetyl palmitate SLN in dependence on polyethylene oxide chain length of the used surfactants. Error bars represent the standard deviation ($n=3$).

A similar relationship was also observed for apoA-IV, adsorption being highest using poloxamers with short PEO chains (Fig. 6). There seems to be a linear correlation between adsorbed apoA-IV and the PEO chain length. When looking at the 2-DE gels, it is noticeable that apoE and apoA-IV have quite similar coordinates on the gel (apoE: pI=5.4–6.1, MW=34,200, apoA-IV: pI=5.05–5.20, MW=46,000). Interesting is the fact that apoA-IV is adsorbed in much higher quantities than apoE. There is also in discussion that apoA-I and A-IV are also involved in brain targeting. In fact, it has been published that high amounts of apoA-IV promote brain uptake [47]. ApoA-I and A-IV are known to suppress the activity of apoE receptors in the liver [48], thus consequently enhancing the uptake via the LDL receptor on the blood–brain barrier.

Another relationship was found for the adsorption of apoJ as a function of the PEO chain length. A highest apoJ adsorption was observed with stabilizing polymers having PEO chain length between approximately 60 and 100 units (i.e. poloxamer 237 and poloxamer 238). Lowest apoJ adsorption took place with 13 PEO units (poloxamer 184) and 121/128 units (poloxamine 908/poloxmer 338) (Fig. 6).

The adsorption of the other apolipoproteins apoA-I, apoC-III and apoA-II did not show any obvious relationship to the chain length of the PEO units. However, remarkable are the proportions of these apolipoproteins among each other, being similar for each polymer (Fig. 7).

As expected, the total amount of adsorbed protein (Fig. 8) shows high adsorption for the SLN stabilized with poloxamer 184, as it is the poloxamer with shortest PEO chains. However, and apart from this, the total amount is very similar with the other poloxamers. Surprisingly, the protein adsorption is also very high with poloxamine 908 (121 PEO units). It is even slightly higher than the total protein adsorption on the lowest MW poloxamer 184. To verify this result, we determined the total mass of adsorbed

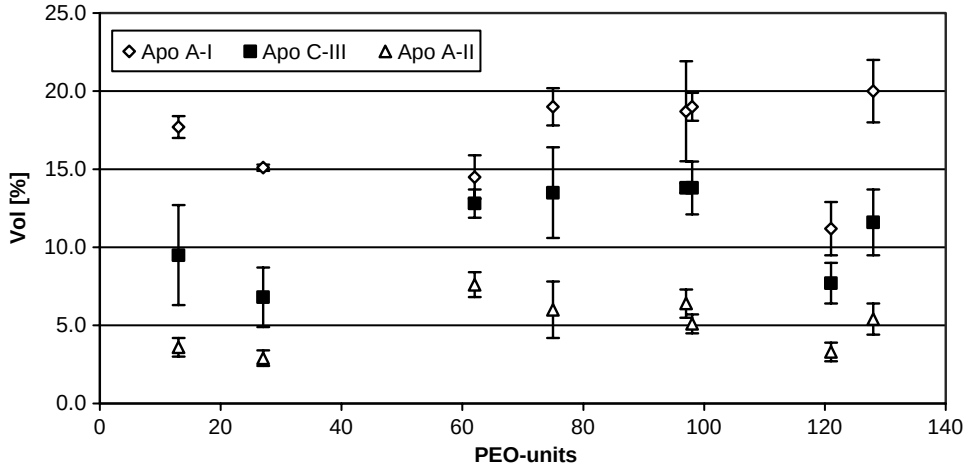

Fig. 7. Percentages adsorbed apoA-I, apoC-III and apoA-II on the cetyl palmitate SLN in dependence on polyethylene oxide chain length of the used surfactants. Error bars represent the standard deviation ($n=3$).

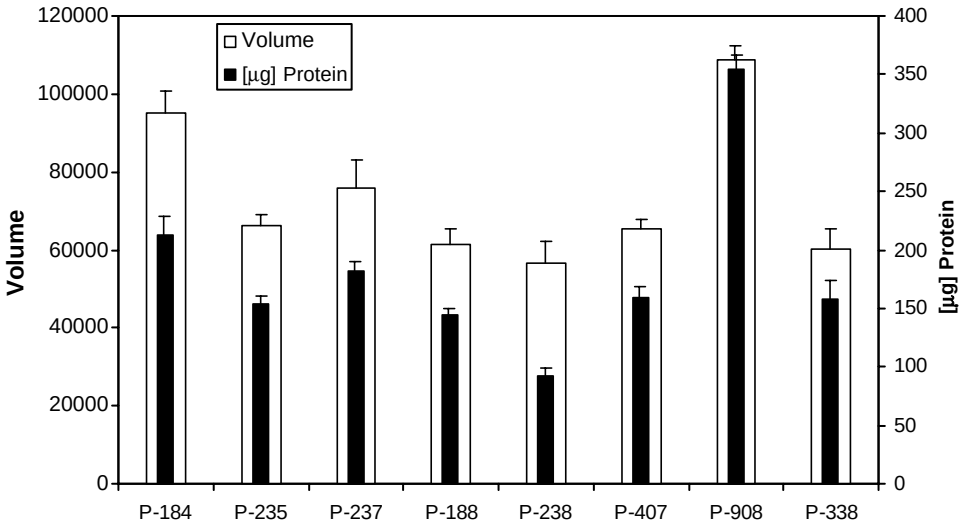

Fig. 8. Total amount of adsorbed proteins on the different cetyl palmitate SLN obtained after 2-DE analysis (white bars, Volume) and BCA-protein assay (black bars, µg). Error bars represent the standard deviation ($n=3$, surface area similar for all particles being approximately 1.3 m^2).

proteins by a BCA-protein assay and our first findings were confirmed (Fig. 8). Table 4 lists the total protein amounts given in micrograms.

As already mentioned, polystyrene 60 nm model particles surface-modified with poloxamine 908 are able to circulate longer in the blood. From this it can be concluded that the total amount of adsorbed protein is not necessarily a parameter to judge the MPS uptake. In general, there is a tendency to say that the less the protein adsorption onto the particles, the less pronounced is the recognition by the MPS. The poloxamine 908 example shows very nicely that despite a relatively high protein adsorption onto the surface, uptake by the MPS is eliminated, meaning that it is just necessary that the 'right' proteins must be adsorbed on the surface. Right proteins are dysopsonins and 'right' is as well the absence of immunoglobulin and complement activating proteins. This is exactly the case of poloxamine 908-coated SLN (1.8% immunoglobulin adsorbed), which means that despite a relatively high protein adsorption, no MPS uptake might occur. Similar results were obtained for 60 nm polystyrene model particles coated with poloxamine 908. They had a protein adsorption of 97.7% apolipoproteins [44]. Of course, it needs to be kept in mind that the slope obtained from silver-staining is different for each protein, limiting a comparison.

4. Conclusion

A remarkable difference in amount and type of proteins adsorbed on SLN stabilized with different block copolymers was found. The interactions between plasma proteins and colloidal surfaces are of a complex nature and a single parameter determining the whole plasma protein pattern could not be established—as expected from a complex biological process. The adsorption patterns depend not only on the accessibility of the surface for the proteins (extent of steric hindrance) but also on other surface properties such as hydrophobicity, presence of complement activating groups or charge density. Nevertheless, SLN stabilized with the low MW poloxamer 184 and poloxamer 235 showed interestingly high adsorption of apoE, the one mediating the uptake across the blood-brain barrier. These SLN also showed highest adsorption of apoA-IV, low adsorption of apoC-II (particularly SLN stabilized with poloxamer 235) and the absence of opsonins. Moreover, previous studies performed by Batrakova et al. demonstrated that both the poloxamer 235 unimer and micelle-mediated transport mechanisms could be used to affect the permeability of drugs across the blood–brain barrier. Based on these findings, we conclude that especially poloxamer 235 stabilized-SLN are promising systems to be used to

Table 4
BCA-protein assay: total protein amounts in µg in the different protein samples obtained from protein adsorption on SLN formulations

Proteins adsorbed	P-184	P-235	P-237	P-188	P-238	P-407	P-908	P-338
µg	213±16	154±6	182±8	145±5	92±7	159±9	354±12	158±15
Volume	95,149±5418	66,170±2855	75,870±7329	61,364±4194	56,539±5477	65,582±2178	108,768±3729	60,110±5111

The protein amounts refer to an incubated surface area of approximately 1.3 m^2, according to the incubated particle surface for the 2-DE experiment (total protein amounts expressed in volume). The values are the mean of three experiments.

deliver drugs to the brain. However, this must be confirmed in further in vitro and in vivo studies. At the same time SLN have the potential to be a regulatory accepted carrier, in opposite to the former analyzed polymeric model carriers. In a long-term development process of such carriers, the knowledge gained from previous studies with polymeric model carriers and from such basic studies in combination with biodistribution studies should be applicable to the controlled design of such biodegradable drug carriers for intravenous drug targeting.

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