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Adsorption kinetics of plasma proteins on solid lipid nanoparticles for drug targeting

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Abstract

The interactions of intravenously injected carriers with plasma proteins are the determining factor for the in vivo fate of the particles. In this study the adsorption kinetics on solid lipid nanoparticles (SLN) were investigated and compared to the adsorption kinetics on previously analyzed polymeric model particles and O/W-emulsions. The adsorbed proteins were determined using two-dimensional polyacrylamide gel electrophoresis (2-DE). Employing diluted human plasma, a transient adsorption of fibrinogen was observed on the surface of SLN stabilized with the surfactant Tego Care 450, which in plasma of higher concentrations was displaced by apolipoproteins. This was in agreement with the "Vroman-effect" previously determined on solid surfaces. It says that in the early stages of adsorption, more plentiful proteins with low affinity are displaced by less plentiful with higher affinity to the surface. Over a period of time (0.5 min to 4 h) more interesting for the organ distribution of long circulating carriers, no relevant changes in the composition of the adsorption patterns of SLN, surface-modified with poloxamine 908 and poloxamer 407, respectively, were detected. This is in contrast to the chemically similar surface-modified polymeric particles but well in agreement with the surface-modified O/W-emulsions. As there is no competitive displacement of apolipoproteins on these modified SLN, the stable adsorption patterns may be better exploited for drug targeting than particles with an adsorption pattern being very dependent on contact time with plasma.

Keywords: Plasma protein adsorption kinetics; Solid lipid nanoparticles; Two-dimensional polyacrylamide gel electrophoresis; Vroman-effect; Poloxamer 407; Poloxamine 908

1. Introduction

Plasma protein adsorption on particulate drug carriers for intravenous injection is generally regarded as the determining factor for the in vivo behavior of nanoparticles and microparticles (Juliano, 1988; Müller and Heinemann, 1989; Price et al., 2001; Thiele

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et al., 2003). For example, binding of opsonins (e.g. immunoglobulin G, complement factors, and fibrinogen) promotes phagocytosis and removal of the particles from the systemic circulation by cells of the mononuclear phagocytic system (MPS) (Leroux et al., 1995; Camner et al., 2002). In contrast, binding of dysopsonins (e.g. albumin) promotes prolonged circulation time in the blood (Moghimi et al., 1993; Ogawara et al., 2004). Moreover, surface-enriched proteins can mediate an uptake of the particles by specific target-cell populations (Kreuter et al., 2002; Müller and Schmidt, 2002). Therefore, the plasma protein adsorption patterns on different carrier systems were analyzed in vitro employing two-dimensional polyacrylamide gel electrophoresis (2-DE) (Blunk et al., 1993; Thode et al., 1997; Harnisch and Müller, 1998; Lück et al., 1998b; Gessner et al., 2001).

In these studies, the particles were incubated routinely for 5 min in citrated plasma and subsequently the adsorbed proteins were eluted from the surface and analyzed by 2-DE. An incubation time of 5 min was chosen because in general the first 5 min after i.v. injection are decisive for the fate of the particles. In case recognition by the MPS occurs, up to 90% of the injected dose are taken up by the liver macrophages (O'Mullane et al., 1987; Liliemark et al., 1995). In case particles "survive" these first 5 min, prolonged blood circulation was found (Illum et al., 1987; Cattel et al., 2003). Therefore, the protein adsorption pattern acquired in the first 5 min is the most important one, determining MPS recognition or MPS escape (at least for a certain time).

When using plasma, it is important to bear in mind that the addition of an anticoagulant such as sodium citrate – which chelates bivalent cations like calcium – leads to inactivation of the amplifying systems such as complement or coagulation (Babensee et al., 1998; Yamazaki et al., 1999). Nevertheless, in this study plasma is a more appropriate incubation medium for evaluating competitive adsorption in vitro than serum, because in many other studies the adsorption of typical plasma proteins, such as fibrinogen, onto different types of particles was demonstrated (Blunk et al., 1993; Lück et al., 1997; Jahangir et al., 2003; Archambault and Brash, 2004; Unsworth et al., 2005) and the adsorption of a single protein strongly depends on the composition of the mixture from which the adsorption appears (Price et al., 2001; Cornelius et al., 2002b). However, when a correlation between the in vivo behavior of particulate drug carriers and their protein adsorption detected in vitro is approached, it is important to consider the protein adsorption patterns obtained after incubation in both plasma and serum. In a strict sense, only investigations using blood can provide expressive answers with regard to adsorption of plasma proteins onto surfaces in vivo.

Protein adsorption onto solid surfaces has been reported to be time-dependent. In fact, the adsorption patterns have to be regarded as a product of a sequence of adsorption of more plentiful proteins with lower affinity and their displacement by less plentiful proteins with higher affinity to the investigated surface (Vroman et al., 1980; Vroman and Adams, 1986). This displacement often occurs within seconds or even within a fraction of a second and this early transient adsorption is called "Vroman-effect". Its extent depends on the surface onto which the proteins are adsorbed (Boisson-Vidal et al., 1991; Brash and Ten Hove, 1993). Blunk et al. (1996) demonstrated such a "Vroman-effect" with polymeric model particles employing different dilutions of plasma. The dilution produces a prolonged resistance time of the plentiful proteins on the surface as the concentration of the lower plenty proteins having a higher affinity to the surface decreases significantly (Vroman and Adams, 1986). However, Harnisch and Müller (2000) observed no competitive displacement of plasma proteins on emulsion droplets and concluded that the differences in the adsorption kinetics are due to the different chemical nature of the systems, the resulting different surface properties and consequently the different binding facilities of the proteins to the surface.

Another interesting carrier system for parenteral drug delivery, showing good tolerability in vivo (Weyhers et al., 1995; Olbrich et al., 2004), is the solid lipid nanoparticles (SLN) (Müller et al., 2000; Müller et al., 2002; Wissing et al., 2004). The 2-DE technique was previously modified and successfully transferred to SLN (Göppert and Müller, 2004). The goal of the first part of this study was to investigate if it also exists a "Vroman-effect" on SLN, as they have a solid matrix as the polystyrene model particles, or if the adsorption kinetics are similar to the emulsion systems as they also have a lipid matrix, i.e. are chemically very similar. Furthermore, the adsorption kinetics of plasma proteins on poloxamer 407-stabilized SLN (P407-SLN) and poloxamine 908-stabilized SLN (P908-SLN) over

a period of time, i.e. 0.5 min to 4 h, were investigated. This should especially be relevant to the in vivo behavior of surface-modified SLN as potential drug carriers for site-specific delivery. Previously, it was shown that polymeric model particles surface-modified with poloxamer 908 circulated in the blood stream (Illum et al., 1987; Moghimi, 1999), while polymeric model particles (Illum and Davis, 1987; Porter et al., 1992) and poly(lactide-co-glycolide) nanospheres (Park et al., 2003), both surface-modified with poloxamine 407, accumulated in the bone marrow. Moreover, Müller et al. (1996) showed that P407-SLN and P908-SLN proved more efficient in avoiding in vitro phagocytosis than polystyrene particles surface-modified with these block copolymers. It is likely, that while circulating in the blood stream a change in adsorption patterns might occur. This will be clarified in the second part of this study.

2. Materials and methods

2.1. Materials

Tego Care 450 (polyglyceryl-3-methylglucose distearate) was provided by Goldschmidt (Essen, Germany), the block copolymers poloxamer 407 (Synperonic F127) and poloxamine 908 (Tetronic 908) from ICI Surfactants (Middlesborough, UK). Cetyl palmitate was purchased from Henkel KG (Düsseldorf, Germany). Citrated human plasma was obtained from the German Red Cross (Berlin, Germany) and stored at −70 °C. Sepharose 2B for gel filtration and Immobiline DryStrips (IPG-strips, pH 3–10, nonlinear) for the first dimension of 2-DE were supplied from Amersham Pharmacia Biotech (Uppsala, Sweden). For 2-DE all chemicals according to Hochstrasser et al. (1988) were of analytical grade and purchased either from BioRad (Munich, Germany) or Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Solid lipid nanoparticles

SLN are particles made from solid lipids (i.e. solid at room temperature and also at body temperature) and stabilized by surfactants. They were produced by the hot high pressure homogenization technique as described previously (Müller and Lucks, 1996). Briefly, the lipid was melted (approximately 5 °C above the melting point of the lipid) and dispersed in an aqueous surfactant solution having the same temperature. A hot pre-emulsion was obtained by high speed stirring using an Ultra turrax T25 (Janke & Kunkel, Staufen, Germany). Subsequently, the hot pre-emulsion was homogenized using a Micron LAB 40 (APV Systems, Unna, Germany), applying three homogenization cycles at 500 bar. The obtained nanoemulsion recrystallized upon cooling to room temperature forming SLN.

Three different systems were produced for protein adsorption kinetics studies. For the first part of the study, “model-SLN” were produced using cetyl palmitate as matrix lipid (10.0%, w/w) and Tego Care 450 as surfactant (1.2%, w/w) (TC-SLN). For the second part two different formulations were prepared, once again cetyl palmitate as matrix lipid (5.0%, w/w) and poloxamer 407 (2.5%, w/w) (P407-SLN), and poloxamine 908 (2.5%, w/w) (P908-SLN) as stabilizers, respectively.

Particle size measurement was performed 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).

2.2.2. Sample preparation

2.2.2.1. Sample preparation for the study of the “Vroman-effect”. To study a possible “Vroman-effect” 500 μl of TC-SLN suspension (mean diameter 220 nm) were incubated in 1500 μl of undiluted citrate-stabilized human plasma (corresponding approximately to 1.1 m^2 surface area/ml plasma) at 37 °C for 5 min (final plasma concentration in the sample: 75%, v/v). Additionally, the same amount of TC-SLN suspension was incubated in 1500 μl of plasma, diluted with double-distilled water to concentrations of 1.6%, 16%, 44%, and 73.3% (final plasma concentrations in the samples: 1.2%, 12%, 33%, and 55% (v/v), respectively). In the study of Blunk et al. (1996), 300 μl of particle suspension (5%, w/v), mean diameter 997 nm) were incubated in 1200 μl of plasma, corresponding approximately 0.075 m^2 surface area/ml plasma.

The particle separation from the incubation medium by centrifugation and the following three washing steps with 20 mM phosphate buffer pH 7.4 were performed as described previously (Harnisch and Müller, 1998).

Briefly, after centrifugation (60 min at 22,940 × *g* using a Biofuge 22 R, Heraeus, Hanau, Germany), the lipid formed a coherent top layer, and the plasma and washing solution, respectively, were removed by use of a syringe. The adsorbed proteins were desorbed by adding a solution containing 10% (w/v) sodium dodecyl sulfate (SDS) and 2.32% (w/v) dithioerythritol (DTE) (5 min at 95 °C) (Cook and Retzinger, 1992). Finally, 190 μl of a solution containing DTE, cholamidopropyldimethylhydroxypropanesulfonate (CHAPS), urea, Tris and bromophenol blue were added and the mixture was stirred and centrifuged for 15 min at 22,940 × *g*. Hundred microlitres of this solution were applied to first dimension of the 2-DE.

2.2.2.2. Sample preparation for a study of the adsorption kinetics over a period of time. In contrast to the centrifugation method used in routine studies (Harnisch and Müller, 1998; Lück et al., 1998a), the gel filtration method (size exclusion chromatography) allows separation of particles with density values too close to the density value of water (Diederichs, 1996; Göppert and Müller, 2004). Moreover, it enables the separation of particles from excess plasma even after the samples had been incubated only for a few seconds. In this part of the study, the incubation process should be finished after 0.5 min. Thus, the gel filtration method according to Göppert and Müller (2004) was used. Briefly, 200 μl of P407- and P908-SLN suspension, respectively, was incubated with 600 μl of plasma and after the specified incubation time, the sample was applied on the top of a 1 cm × 20 cm column from Amersham Pharmacia Biotech, filled with a bed volume of 10 ml of Sepharose 2B. Afterwards, the column was flushed at a flow rate of 18 ml/h with 10 mM phosphate buffer pH 7.4. Sample fractions were collected in sub volumes of 1.0 ml each and UV absorbance determined using an Uvikon 940 Photometer (Kontron instruments, Eching, Germany). Detection wavelengths were 279 nm (absorption maximum of plasma) and 350 nm (in contrast to particle suspension, no absorption of plasma at this wavelength). In size exclusion chromatography elution starts with the largest particles, from this the SLN (P407-SLN 236 nm, P908-SLN 212 nm) elute before the plasma proteins. Using a two-equation system, the calculation of the concentration of each component in each fraction was possible (Thode and Müller, 1997), and fraction 3 and 4 were determined as being plasma free and therefore chosen to be used for 2-DE analysis. Elution of the adsorbed proteins and the following step were performed as described in Section 2.2.2.1. A comparison with the adsorption patterns obtained using centrifugation as separation method showed that there was no change in the resulting patterns (Göppert and Müller, 2004).

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

Electrophoretic analysis was performed essentially as described by Hochstrasser et al. (1988) for standard plasma samples. Later the protocol was modified and successfully transferred to colloidal drug carriers by Blunk et al. (1993). Briefly, in the first dimension (isoelectric focussing, IEF) the proteins are separated according to their isoelectric points (IP). IEF was carried out with IPG-strips in a Multiphore II from Amersham Pharmacia Biotech equipped with a E 752 power supply from Consort (Turnhount, Belgium). After equilibration of the IPG-strips (Bjellqvist et al., 1993), the proteins are separated on the basis of their molecular weights (MW) in the second dimension of 2-DE (SDS-PAGE). For SDS-PAGE the Protean II Multi-Cells with a 1000 V/500 mA power supply, both from Bio-Rad, were used according to Görg et al. (1995). After electrophoretic separation the gels were silver-stained (Bjellqvist et al., 1993) and scanned with an ImageScanner from Amersham Pharmacia Biotech. The spots were identified by matching the gels with master maps of human plasma (Anderson and Anderson, 1991; Hoogland et al., 2000) and the data were processed using the MELANIE III software from Bio-Rad.

3. Results and discussion

3.1. Early protein adsorption ("Vroman-effect") on SLN

Vroman has postulated a fast sequence of protein adsorption and desorption on solid surfaces, where more plentiful proteins are displaced by less plentiful (Vroman et al., 1980; Vroman and Adams, 1986). The replacement of one protein by another species might take place in a split second. This phenomenon is called

"Vroman-effect", especially for transiently adsorbed fibrinogen. If particle separation from excess plasma employing gel filtration or even more when using centrifugation is time consuming, the initially adsorbed proteins are not detected at all. However, by diluting the plasma sufficiently, the concentration of the displacing proteins with higher affinity would be decreased to such an extent, that the resistance times of the plentiful proteins which adsorb first would be prolonged, and therefore these proteins will become detectable (Brash, 1987; Brash and Ten Hove, 1993). Thus, adsorption from different dilutions of plasma was investigated to determine the very early stages of the adsorption process. As the extent of the "Vroman-effect" depends on the surface onto which the proteins are adsorbed, it was important to maintain the same ratio of plasma volume to surface area as used in the former studies with polymeric model particles and emulsions, respectively. For the polymeric particles, Blunk used dilutions of 0.08%, 0.8%, and 80% plasma (Blunk et al., 1996). Due to the approximately 15-fold higher amount of particle surface area in the SLN suspension compared to the polymeric particles (1.1 m^2/ml plasma versus 0.075 m^2/ml), the alignment led to values of 1.2%, 12%, and due to the feasibility a maximum of 75% plasma concentration in the sample (explained by the SLN to plasma ratio of 1:3 used for routine analysis).

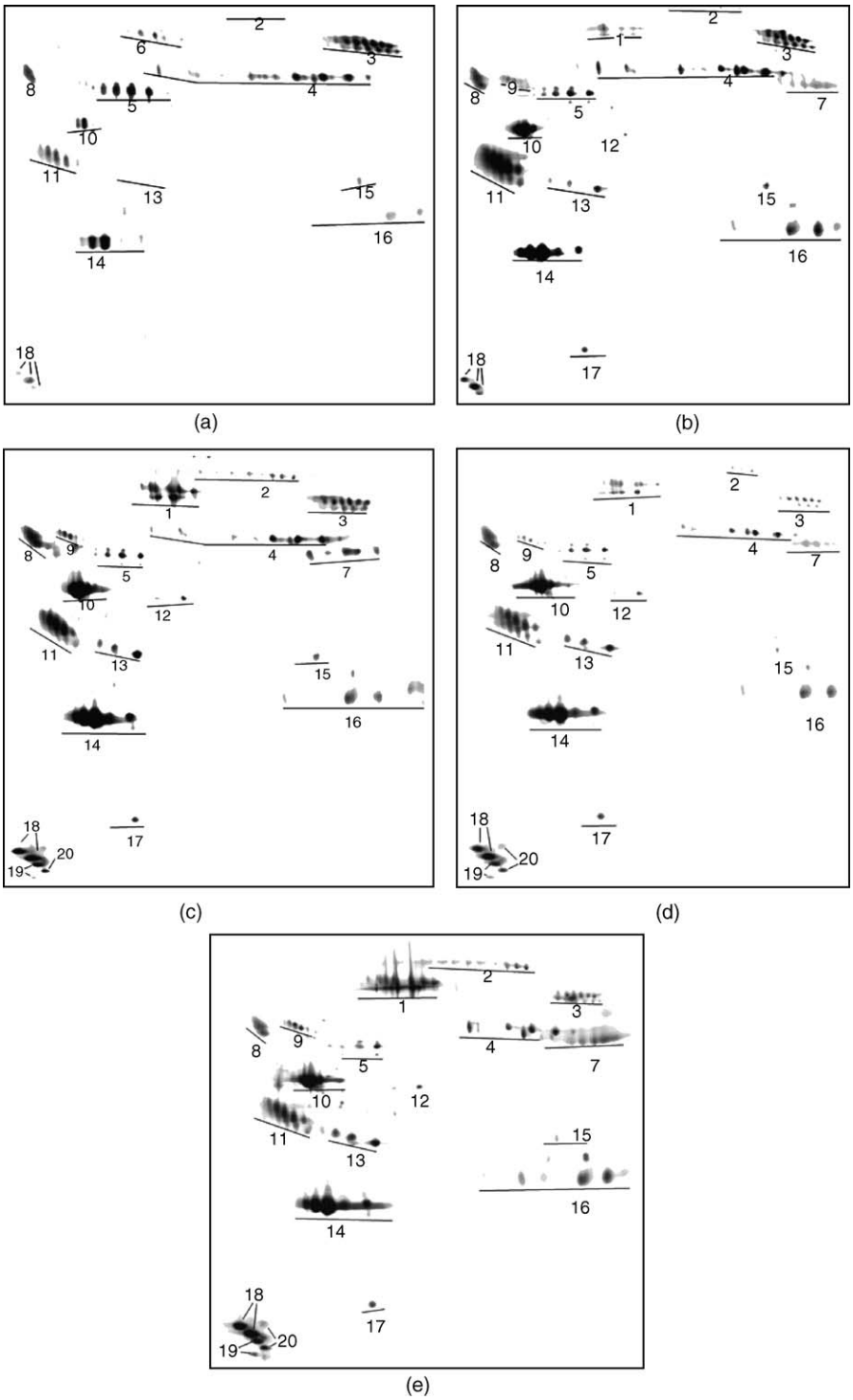
In contrast to the solid surface of polymeric model particles, Harnisch and Müller obtained no protein adsorption on emulsions incubated with a dilution of 1.1% plasma and only a few proteins adsorbed when incubated with 11% plasma (Harnisch and Müller, 2000). Once this result appeared also possible when using SLN suspensions, intermediate concentrations of 33% and 55% of plasma were additionally used as incubation media.

Blunk et al. observed that the most abundant protein in human plasma (albumin, 3500–5000 mg 100 ml^{-1}) was displaced by fibrinogen (200–450 mg 100 ml^{-1}), which in turn was displaced by IHRP (inter-α-trypsin inhibitor family heavy chain-related protein; concentration in plasma not known exactly, but markedly lower than fibrinogen), apoC-III (12–14 mg 100 ml^{-1}) and apoJ (3.5–10.5 mg 100 ml^{-1}) (Blunk et al., 1996).

However, Harnisch and Müller observed that with increasing plasma concentration the amount of the major proteins (particularly apolipopoteins and to a lesser extent immunoglobulin chains, and albumin) adsorbed on emulsion droplets increased steadily while their percentage, related to the overall amount of adsorbed proteins, remained nearly unchanged (Harnisch and Müller, 2000). There was even no fibrinogen detectable when using 1.1% plasma dilution.

Fig. 1 shows the whole 2-DE gels obtained with TC-SLN from incubation with 1.2%, 12%, 33%, 55%, and 75% plasma solution. The five adsorption patterns resulted from an incubation time of 5 min each. Fig. 2a shows the amounts of the major proteins adsorbed from the different plasma dilutions and in Fig. 2b the percentages of the major proteins detected on the 2-DE gels are compared. On the adsorption pattern from 1.2% plasma (Fig. 1a) fibrinogen was present in the highest amount (60% of the overall amount of adsorbed proteins, Fig. 2b). With increasing plasma concentrations the amounts of fibrinogen steadily decreased (to 6.8% fibrinogen with 75% plasma), while the amount of the apolipoproteins steadily increased (from 25% of the overall amount with 1.2% plasma to 68% of the overall amount with 75% plasma). Therefore, in agreement with Vroman et al. (1980), Brash and Ten Hove (1993) and Blunk et al. (1996) fibrinogen seemed indeed to have been adsorbed transiently on the solid particles. The concentrations of the several apolipoproteins in human plasma are markedly lower than that of fibrinogen, but their affinity to (especially hydrophobic) surfaces is much higher. It has been reported that apolipoproteins show a relatively flexible molecular structure, able to change their conformation when adsorbing onto surfaces ("soft proteins") (Graham and Phillips, 1979). This ability of changing its multiple α-helical segments in the interface in contact with water is the crucial property of these proteins for the efficient removal of non-polar residues, which cannot be achieved in the molecule of "hard proteins"

Fig. 1. Plasma protein adsorption patterns of TC-SLN after incubation with 1.2% (a), 12% (b), 33% (c), 55% (d), and 75% plasma solution (e). 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) IgM μ chain, (3) fibrinogen α, (4) fibrinogen β chain, (5) fibrinogen γ chain, (6) IgD δ chain, (7) IgG γ chain, (8) α2-HS-glycoprotein, (9) α1-antitrypsin, (10) apoA-IV, (11) apoJ, (12) haptoglobin-β-chain, (13) apoE, (14) apoA-I, (15) C4γ, (16) Ig light chain, (17) transthyretin, (18) apoC-III, (19) apoC-II, (20) apoA-II.


(a)
(b)
(c)
(d)
(e)

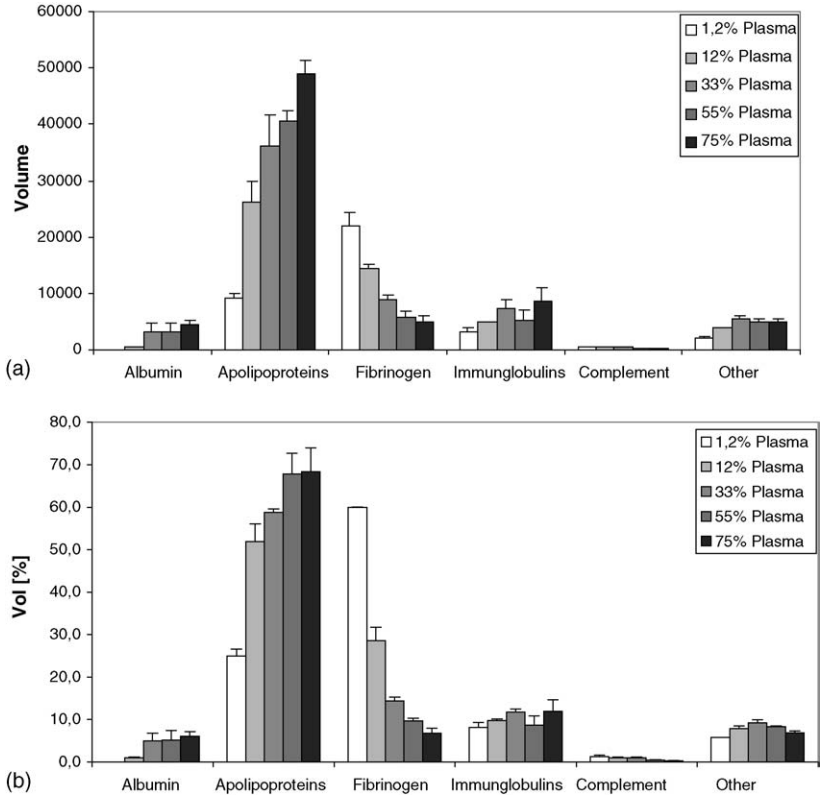


Fig. 2. Total amounts (a) and percentages of the major proteins (b) adsorbed from the different plasma dilutions on TC-SLN (error bars represent the standard deviation ($n = 2$)).

(e.g. fibrinogen or albumin) (Arai and Norde, 1990; Phillips, 1992). A nice and recently investigated example is apoA-I. It was shown that apoA-I is a significant component of the protein layer adsorbed from plasma to a variety of biomaterial surfaces, e.g. liposomes or hydrophobic and even hydrophilic polymers (Cornelius et al., 2002a). The authors of this study concluded that apoA-I deposition may be an important effect in blood-biomaterial interactions generally. ApoA-I is the major apolipoprotein of high density lipoproteins (HDL) and this lipid-bound state is thermodynamically favored (Rosseneu, 1992). However, like the other apolipoproteins, apoA-I is only weakly associated with the lipoprotein surface and may transfer from one lipoprotein particle to another (Rosseneu, 1992). Therefore, in case of plasma contact with nanoparticles, it is likely that apoA-I may transfer from the HDL surface to the nanoparticles surface. This is likely in particular for hydrophobic surfaces such as SLN or polystyrene model particles, because apoA-I can adsorb in his thermodynamically favored state.

Therefore, in plasma of high concentration, fibrinogen is displaced within seconds or even fractions of a second by apolipoproteins.

Surprisingly, the adsorption behavior of albumin was different. Albumin is by far the most abundant protein in plasma, but in contrast to the polymeric model particles (Blunk et al., 1996) it is not adsorbed immediately after plasma contact. By diluting the plasma to 1.2%, neither with emulsions (Harnisch and Müller, 2000) nor SLN suspensions, a transient adsorption of albumin could be demonstrated. On the contrary, albumin seemed to have similar adsorption behavior like apolipoproteins. The former was first detected in small amounts with 12% plasma (1% of the overall protein amount) and the highest amount (6%) appeared with 75% plasma. Nevertheless, these data confirmed

Table 1
Total amount (vol) and volume percentages (vol (%)) of the most abundant protein species adsorbed onto P407-SLN, and P908-SLN, respectively (values are the means of two experiments, ± are the standard deviations)

Adsorbed protein	System	P407-SLN				P908-SLN			
		0.5 min[a]	5 min[a]	30 min[a]	240 min[a]	0.5 min[a]	5 min[a]	30 min[a]	240 min[a]
Albumin	vol	337 ± 61	806 ± 138	1713 ± 159	1889 ± 182	835 ± 33	1261 ± 85	1894 ± 178	3829 ± 310
	vol (%)	2.9 ± 0.4	3.8 ± 0.7	7.6 ± 1.0	7.6 ± 1.1	6.0 ± 0.1	7.7 ± 0.7	9.6 ± 1.6	14.70.6
apoA-I	vol	2529 ± 83	2183 ± 382	3024 ± 380	2812 ± 169	3749 ± 293	3854 ± 248	5714 ± 384	5945 ± 161
	vol (%)	21.3 ± 1.0	10.4 ± 1.8	13.2 ± 1.4	11.3 ± 1.3	26.8 ± 0.6	23.5 ± 1.0	28.6 ± 0.4	22.9 ± 0.4
apoA-II	vol	423 ± 2	888 ± 215	1213 ± 187	1437 ± 457	523 ± 82	577 ± 64	410 ± 237	587 ± 76
	vol (%)	3.5 ± 0.1	4.2 ± 1.0	5.3 ± 0.7	5.7 ± 1.5	3.7 ± 0.4	3.5 ± 0.3	2.0 ± 1.0	2.2 ± 0.4
apoA-IV	vol	676 ± 43	971 ± 5.9	1221 ± 174	1278 ± 133	1036 ± 54	1954 ± 71	1862 ± 141	3112 ± 361
	vol (%)	5.7 ± 0.4	5.9 ± 0.1	5.3 ± 0.6	5.1 ± 0.3	7.5 ± 0.1	11.9 ± 0.7	9.4 ± 1.5	11.9 ± 0.9
apoC-II	vol	738 ± 29	1421 ± 107	1452 ± 46	1757 ± 480	916 ± 308	1204 ± 167	1459 ± 123	1060 ± 139
	vol (%)	6.2 ± 0.2	6.7 ± 0.5	6.4 ± 0.1	7.0 ± 1.6	6.5 ± 1.8	7.4 ± 0.8	7.3 ± 0.0	4.1 ± 0.4
apoC-III	vol	2746 ± 322	5962 ± 464	6529 ± 636	6278 ± 21	3003 ± 22	3312 ± 132	3302 ± 374	4275 ± 137
	vol (%)	23.1 ± 2.3	28.3 ± 2.1	28.6 ± 2.2	25.2 ± 1.4	21.6 ± 1.1	20.2 ± 0.3	16.5 ± 0.5	16.5 ± 0.3
apoJ	vol	664 ± 43	1861 ± 193	2449 ± 341	3595 ± 736	2427 ± 226	2754 ± 73	3563 ± 442	5223 ± 174
	vol (%)	5.6 ± 0.4	8.8 ± 1.0	10.7 ± 1.3	14.4 ± 2.2	17.5 ± 2.6	16.8 ± 0.0	17.8 ± 0.8	20.1 ± 0.3
Transthyretin	vol	450 ± 112	634 ± 80	893 ± 50	2123 ± 254	1265 ± 107	1008 ± 16	1079 ± 268	1126 ± 121
	vol (%)	3.8 ± 1.0	3.0 ± 0.4	3.9 ± 0.3	8.5 ± 0.6	9.1 ± 0.2	6.2 ± 0.1	5.4 ± 0.9	4.3 ± 0.7
IgG γ chain	vol	453 ± 75	444 ± 86	192 ± 238	320 ± 124	n.d.	n.d.	n.d.	n.d.
	vol (%)	3.8 ± 0.6	2.1 ± 0.4	0.8 ± 1.1	1.3 ± 0.5	n.d.	n.d.	n.d.	n.d.
Ig light chain	vol	1289 ± 127	2472 ± 72	1254 ± 199	808 ± 29	n.d.	n.d.	n.d.	n.d.
	vol (%)	10.8 ± 0.9	11.7 ± 0.4	5.5 ± 1.0	3.2 ± 0.1	n.d.	n.d.	n.d.	n.d.
IgM μ chain	vol	860 ± 66	1861 ± 163	606 ± 135	696 ± 69	n.d.	n.d.	n.d.	n.d.
	vol (%)	7.2 ± 0.7	8.8 ± 0.8	2.7 ± 0.6	2.8 ± 0.4	n.d.	n.d.	n.d.	n.d.
α1-Antitrypsin	vol	397 ± 54	833 ± 279	1380 ± 49	1043 ± 48	200 ± 112	429 ± 146	705 ± 121	845 ± 90
	vol (%)	3.3 ± 0.5	4.0 ± 1.3	6.0 ± 0.3	4.2 ± 0.4	1.4 ± 0.7	2.7 ± 1.0	3.5 ± 0.3	3.2 ± 0.2
Haptoglobin-β-chain	vol	n.d.	n.d.	480 ± 89	224 ± 135	n.d.	n.d.	n.d.	n.d.
	vol (%)	n.d.	n.d.	2.1 ± 0.4	0.9 ± 0.6	n.d.	n.d.	n.d.	n.d.
Total amount	vol	11903 ± 195	20809 ± 302	22852 ± 476	24940 ± 1293	13954 ± 784	16353 ± 397	19989 ± 1632	26002 ± 1174

[a] Incubation time.

in principle the findings of Vroman et al. (1980) and those of Blunk et al. (1996), that in the very early stages of protein adsorption (strongly diluted plasma) a rapid adsorption of more plentiful proteins and a subsequent displacement by less plentiful ones takes place, also for the carrier system TC-SLN.

3.2. Adsorption kinetics on surface-modified SLN over a period of time relevant to drug targeting

Although protein adsorption after seconds or even a split second is interesting from the academic point of view, it is more important for intravenously injected drug carriers with prolonged circulation time and possible subsequent site-specific drug delivery to investigate the adsorption over a period of minutes and hours. As poloxamine 908 and poloxamer 407 are efficient surface modifiers to reduce MPS uptake (Moghimi et al., 1993; Moghimi, 1999) and poloxamer 407 even to achieve targeting to the bone marrow (Illum and Davis, 1987; Park et al., 2003), the plasma protein adsorption patterns of P407-SLN and P908-SLN, respectively, were determined over a period of time.

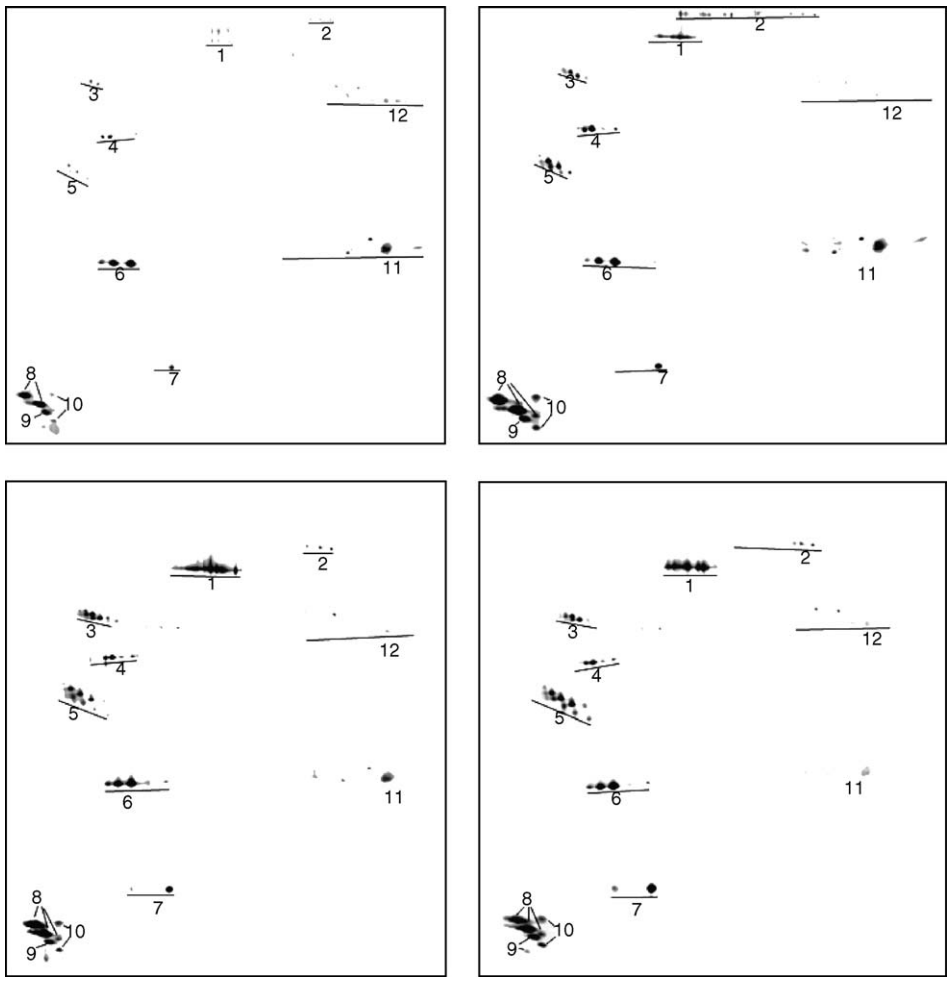


Fig. 3. Plasma protein adsorption patterns of P407-SLN after different incubation time. Top left, 0.5 min; top right, 5 min; bottom left, 30 min; bottom right, 240 min. The entire gels are shown, p*I* 4.0–9.0 (from left to right, non-linear), MW 250–6 kDa (top to bottom, non-linear). (1) Albumin, (2) IgM μ chain, (3) α1-antitrypsin, (4) apoA-IV, (5) apoJ, (6) apoA-I, (7) transthyretin, (8) apoC-III, (9) apoC-II, (10) apoA-II, (11) Ig light chain, (12) IgG γ chain.

Table 1 lists the total amount and volume percentages of the most abundant protein species adsorbed on P407-SLN and P908-SLN, respectively. Fig. 3 shows the 2-DE gels obtained from incubation of P407-SLN with undiluted citrated plasma for 0.5 min (upper, left), 5 min (upper, right), 30 min (lower, left) and 240 min (lower, right). At the first glance it is obvious that once again the apolipoproteins represented the main type of adsorbed proteins. Independently of the incubation time, apoC-III was the most dominant protein on P407-SLN. ApoA-I, apoA-IV, apoJ and apoC-II were also present in a relatively high amount. In addition, albumin, transthyretin, α1-antitrypsin and immunoglobulin chains were detectable in considerable amounts. However, in contrast to TC-SLN, no fibrinogen was detected on P407-SLN. There was even no (transiently) adsorption of fibrinogen detectable after 0.5 min.

Fig. 4a shows the total amounts of protein adsorbed on P407-SLN and Fig. 4b the total amounts of protein adsorbed on P908-SLN. Striking is the fact that with increasing incubation time, the total amount of adsorbed protein steadily increased (P407-SLN 11,900 to 24,940, P-908-SLN 13,950 to 26,000). We anticipated that this increase might be due to a surface degradation of the SLN in plasma and the resulting increase of particles surface. However, PCS measurements (Table 2) revealed that there was only a slightly decrease of particle size after 5 min incubation time. Subsequently, no further decrease of particle size was obtained. A possible explanation for the stability of particle size in plasma after 5 min incubation time might be a protection of the surface against degradation my means of hydrophilic proteins like albumin and apolipoproteins adsorbed on the surface. These adsorbed proteins also led to an increase in particle size. Due to the overlay in particle size decrease and increase, the particles size distribution became broader (Table 2, increasing PI values). However, the increase in particles surface due to particle degradation is not so high to explain the increase in the total amount of protein adsorbed. Thus, we conclude that with increasing incubation time, further proteins will be adsorbed onto the first layer of adsorbed proteins.

Besides the increase in the total amount of protein adsorbed, the amount of adsorbed apoC-III, apoC-II, and apoA-IV on P407-SLN steadily increased (Fig. 5a, Table 1) while their percentage, related to the overall amount of adsorbed proteins, remained nearly unchanged (Fig. 5b, Table 1). On the other hand, the total amount and percentage of apoJ, apoA-II, albumin and transthyretin steadily increased and the percentage of apoA-I decreased while its total amount remained nearly unchanged.

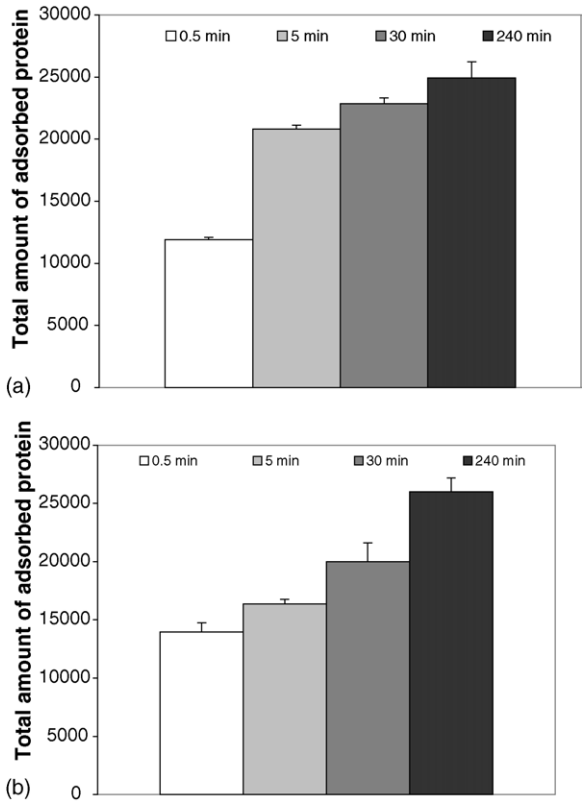


Fig. 4. Total amounts of proteins adsorbed on P407-SLN (a), and P-908-SLN (b) after different incubation time (error bars represent the standard deviation ($n = 2$)).

Table 2
Mean diameter (PCS: photon correlation spectroscopy), calculated surface, and polydispersity index (PI) of poloxamer 407-stabilized SLN (P407-SLN), and poloxamine 908-stabilized-SLN (P908-SLN) in dependency of incubation time in human plasma

		Incubation time			
		0 min	5 min	30 min	240 min
P407-SLN	PCS (nm)	236 ± 3	225 ± 4	225 ± 4	223 ± 4
	Surface (m^2)	0.777	0.815	0.815	0.822
	PI	0.094	0.106	0.122	0.115
P908-SLN	PCS (nm)	212 ± 4	204 ± 5	202 ± 3	205 ± 2
	Surface(m^2)	0.865	0.899	0.908	0.895
	PI	0.120	0.120	0.158	0.139

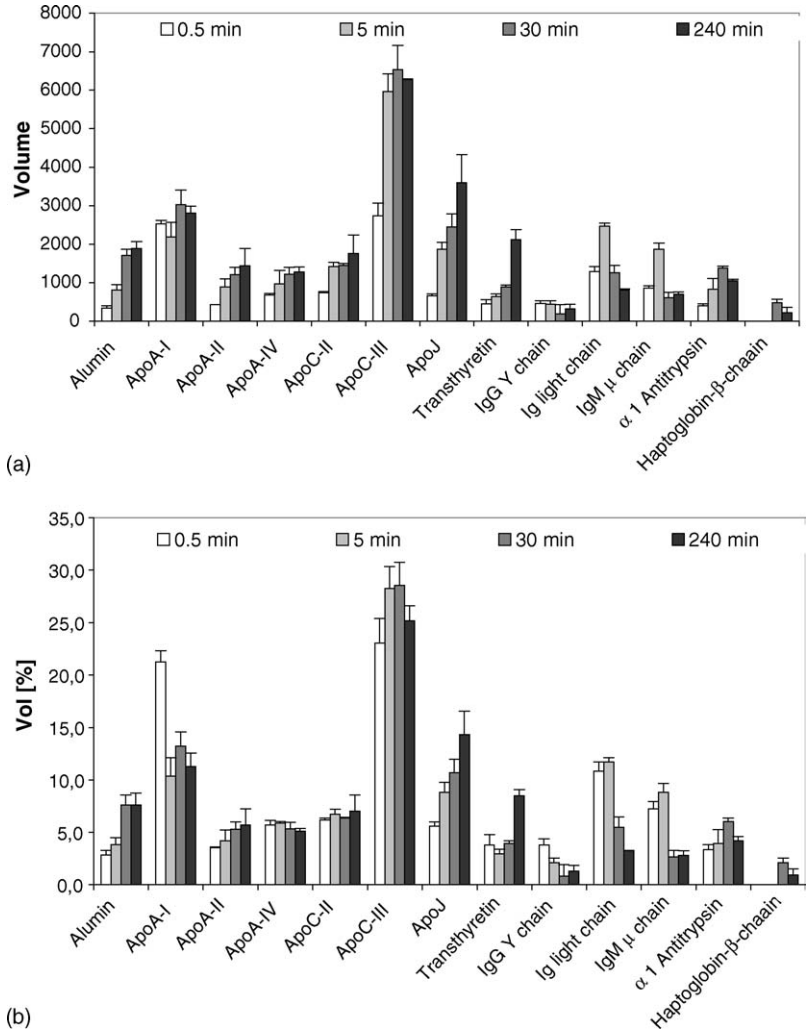


Fig. 5. Total amounts (a) and percentages of the most abundant proteins (b) adsorbed on P407-SLN after different incubation time (error bars represent the standard deviation ($n = 2$)).

Comparison to the adsorption kinetics obtained with P908-SLN (gels not shown) showed further similarities. Once again, there was no fibrinogen detectable. The amount of apoC-III, apoC-II, apoA-IV and here also apoA-I and apoJ steadily increased (Fig. 6a) while their percentage remained nearly unchanged (Fig. 6b). The amount and percentage of albumin and α1-antitrypsin steadily increased with increasing incubation time and the amount and percentage of apoA-II remained nearly unchanged.

However, the polymeric model particles, surface-modified with poloxamer 407 showed a different adsorption behavior (Blunk et al., 1996). The total protein amounts on the gels with incubation time from 0.5 min to 240 min differed only slightly. The patterns did not differ greatly with regard to the qualitative aspects of the adsorbed proteins, but particularly the apolipoproteins showed distinct quantitative changes in the adsorbed amounts. ApoC-III decreased drastically, from 18% after 0.5 min to below 1% after 240 min. The amount of apoE and apoA-I (0.1%, 2%, respectively after 0.5 min) increased to 9% and 8.5%, respectively. On the whole, the adsorption kinetics obtained with polymeric model particles, surface-modified with

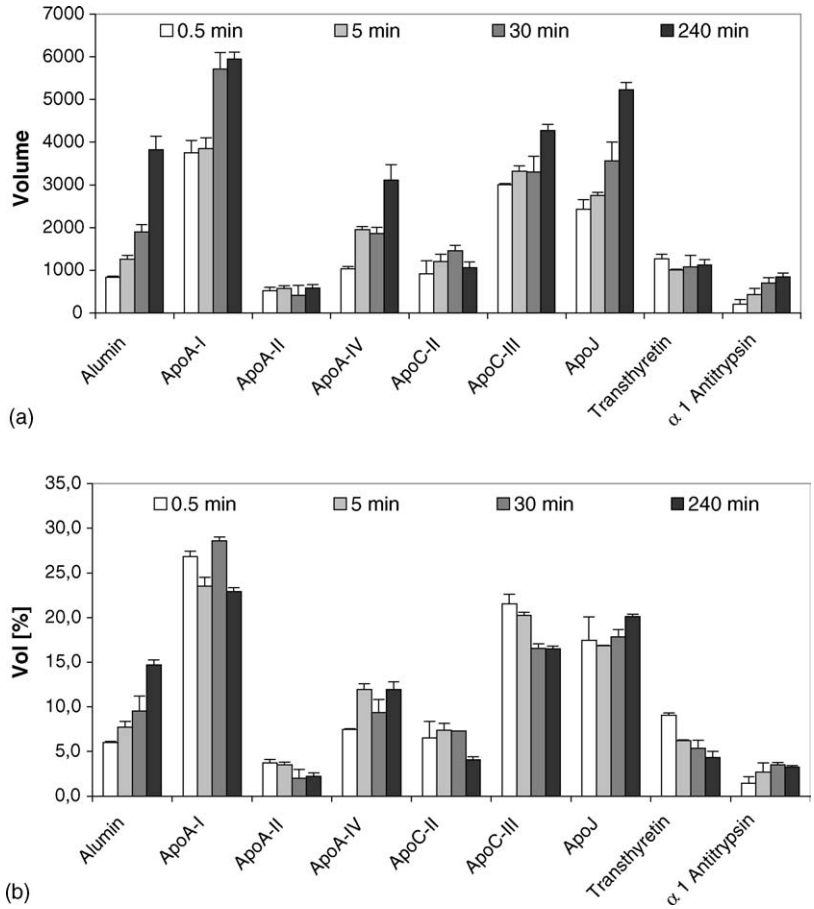


Fig. 6. Total amounts (a) and percentages of the most abundant proteins (b) adsorbed on P908-SLN after different incubation time (error bars represent the standard deviation ($n = 2$)).

poloxamine 908 were in principle the same as with poloxamer 407 (Blunk et al., 1996).

Obviously, there was a displacement of certain proteins by others, however, this time among the apolipoproteins. The changes in the adsorption patterns were drastically, e.g. the ratio apoC-III/apoE dropped from 181 after 0.5 min to 0.09 after 240 min. It is unambiguous that such changes could easily lead to variations in the in vivo behavior and organ distribution of these carriers.

To summarize, the plasma protein adsorption patterns of P407-SLN and P908-SLN are much more stable, than that of chemically similar surface-modified polymeric nanoparticles. On the whole, an increase in incubation time results in an increase of the total amount of adsorbed proteins, but not in the composition of the adsorbed protein layer. No protein desorption from the SLN surface occurred. This observation is in agreement with the former investigated surface-modified O/W-emulsions (Harnisch and Müller, 2000).

4. Conclusion

2-DE in combination with particle incubation in diluted plasma demonstrated the existence of the "Vroman-effect" of transiently adsorbed fibrinogen onto solid surfaces, such as polymeric model particles or TC-SLN. More plentiful fibrinogen was displaced by less plentiful apolipoproteins, having a higher affinity to the surface.

However, the similarities of polymeric model particles and SLN in adsorption kinetics of plasma proteins was restricted to this point (protein adsorption in the very early stages). Investigation of protein adsorption kinetics over a period of minutes and hours revealed that no protein desorption from P407-SLN and P908-SLN occurred. In contrast to the surface-modified polymeric particles, the composition of the adsorption patterns of these SLN seemed to be more stable. Contact with plasma over 4 h entirely led to an increase of the total amount of the proteins adsorbed, as it was previously observed with chemically similar surface-modified O/W-emulsions. A possible explanation for the differences in the adsorption kinetics of these two solid surfaces can be the different nature of the systems and the different binding facilities, especially of the apolipoproteins to the lipid surface.

Thereby, the less pronounced time dependence of protein adsorption on surface-modified SLN might be absolutely beneficial for controlled drug delivery and drug targeting, because the adsorption pattern may therefore be better utilized for this purpose than an adsorption pattern being very addicted on contact time with plasma proteins. This is particularly appropriate for drug carriers having a long circulation time in the blood stream. However, it is important to bear in mind that in citrated plasma we used for evaluating competitive adsorption in vitro, the amplifying systems are blocked. Therefore, to determine possible complement activation or coagulation and to predict the in vivo fate of particulate drug carriers, it is important to consider also the protein adsorption kinetics in blood or at least in both plasma and serum. These issues will be pursued in our future work.

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