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Influence of particle shape on plasma protein adsorption and macrophage uptake

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The purpose of this study was to evaluate the plasma protein adsorption behavior onto different LIPOMER nanoparticles, especially looking for the first time, if the particle shape affects the protein adsorption pattern. The potential *in vivo* fate is discussed and compared with previous *in vivo* animal studies. The two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used for identification of adsorbed plasma proteins. Qualitative similar patterns were obtained from the protein adsorption analysis and four apolipoproteins with considerable quantitative differences were identified. Besides the quantitative differences in the adsorbed apolipoproteins, *in vitro* uptake in the human macrophage cell line U-937 of histocytic lymphoma organ revealed significantly lower uptake of the irregular glycerol monostearate LIPOMER nanoparticles. Therefore, protein adsorption does not seem to play a role in the splenotropic behavior in the sense, that adsorption of opsonins, especially spleen-specific opsonins are required for the uptake. The splenotropic uptake might be favored because all LIPOMER nanoparticles did not adsorb opsonins at all, mediating competitive uptake by liver macrophages. Differences in the *in vivo* uptake by the spleen were attributed to differences in particle shape with potential super position effect by the quantitative differences in the adsorbed proteins.

1. Introduction

Specific targeting of intravenously applied drug delivery systems is more than ever the object in an advanced treatment of diseases. Until now, numerous promising active agents towards many diseases are known, but the delivery to and only to the desired site of action is still the impediment of a more efficient therapy. Often it coincides with considerable side effects. Reduction of side effects is thus another approach of purposeful drug delivery. A comprehensive understanding of all parameters determining the *in vivo* behavior of intravenously injected particles is essential to design drug carriers fulfilling the requirements of a controlled delivery of drugs to the desired site of action.
A challenging task in drug delivery is to prevent intravenously injected particles from recognition and elimination by the cells of the mononuclear phagocytic system (MPS). The MPS is part of the organism's immune defense towards exogenous pathogens. Since nanosized drug delivery systems by i.v. injection into the blood stream immediately interact with human blood proteins, the adsorbed proteins undertake a significant role in the recognition by macrophages and thus, in the further biodistribution of the particles. These considerations led to the concept of differential protein adsorption (Müller and Heinemann 1989). Based on the physicochemical properties of intravenously injected particles certain blood proteins will adsorb onto the surface of the particles, which in turn determine their *in vivo* fate and the organ distribution.
The state of the art technique to analyze the adsorbed proteins on i.v. drug carriers is the two-dimensional polyacrylamide gel electrophoresis (2-D PAGE, 2DE). It was first developed independently by Klose and O'Farrell in 1975 (Klose 1975; O'Farrell 1975) and has then been modified and successfully transferred to analyze the protein adsorption on nanosized drug carriers (Blunk et al. 1993; Harnisch and Müller 1998; Lück et al. 1997). The major advantage of this method is the simultaneous separation and identification of up to 10.000 proteins (Marcus et al. 2009).
Targeting of drug carriers to the spleen is very important as many intracellular infections, like splenic tuberculosis (Imani Fooladi et al. 2009) or trypanosomes (Melo and Brener 1978), are preferentially localized in the spleen. As mentioned above, the main hurdle of targeting to the spleen is the high uptake of foreign particles by Kupffer cells of the liver (Bakker-Woudenberg et al. 1988). In order to circumvent early elimination, adsorption of opsonins, proteins enhancing the uptake by liver macrophages, should be avoided. Furthermore, prolonged circulation times have been reported for PEGylated particles. Poly(ethylene glycol) (PEG) is a hydrophilic polymer which decreases protein deposition and complement activation (Hamad et al. 2010; Vonarbourg et al. 2006). Contrarily, another approach to target particles tissue-specific into the spleen is to engineer particles of poly(vinylpyrrolidone-*co*-vinyl laurate), a hydrophobic polyvinylpyrrolidone (PVP) derivate. Thereby, a long retention in blood circulation was achieved and accompanied by a high splenic accumulation (Yoshioka et al. 2004).

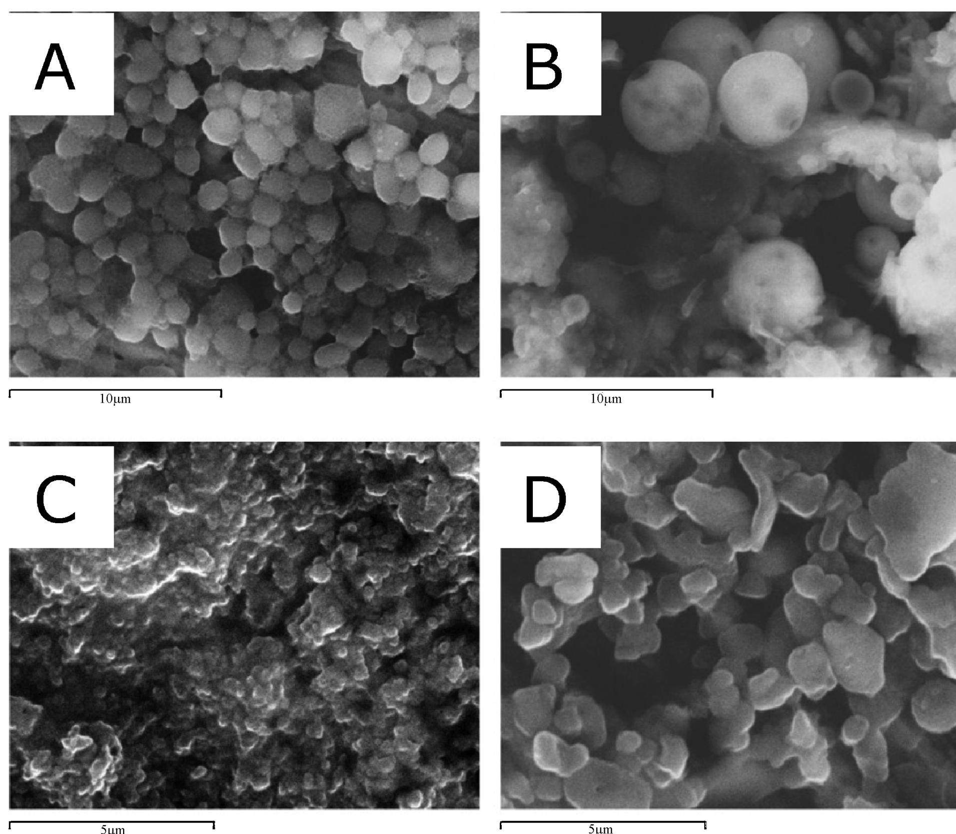

Fig. 1: SEM images of A) sGPS LIPOMER nanoparticles, B) sSA LIPOMER nanoparticles, C) sGMS LIPOMER nanoparticles and D) iGMS LIPOMER nanoparticles

In the present study different polymer lipid (LIPOMER) nanoparticles have been analyzed according to their protein adsorption and a possible *in vivo* fate of the nanoparticles will be discussed. In addition, the potential of a specific splenotropic behavior of irregular shaped glycerol monostearate (iGMS) LIPOMER nanoparticles will be analyzed based on the protein adsorption patterns obtained. Recently, these iGMS LIPOMER nanoparticles have shown macrophage-evading characteristics leading to high splenic concentrations in an animal *in vivo* study which in addition has been confirmed by an *in vitro* phagocytic uptake study (Devarajan et al. 2010). The particle shape has been regarded as a new design parameter leading to the splenotropic targeting behavior. Thus, it is important to confirm, if the particle shape has an influence on the resulting protein adsorption pattern and moreover, if the spleen targeting potential of iGMS LIPOMER nanoparticles can be explained with the adsorbed protein corona.

2. Investigations, results and discussion

2.1. Characterization of LIPOMER nanoparticles

Nanoparticles were prepared by a nanoprecipitation method wherein rapid diffusion of the organic phase in the water was followed by evaporation promoting the formation of nanosized particles. Particle size and entrapment efficiency of LIPOMER nanoparticles were found to be in the range of 350–450 nm and 75–85% (Table). There was no significant difference observed in the particle size and entrapment efficiency after changing the lipid and the non-solvent phase ($p > 0.05$ ANOVA). However, GMS LIPOMER nanoparticles revealed a variation in the shape with a change of the non-solvent phase. While spherical glycerol monostearate (sGMS) LIPOMER nanoparticles were obtained with water as non-solvent, a non-solvent composition water/isopropyl alcohol (IPA) in ratio of 1:1 resulted in the formation of iGMS LIPOMER nanoparticles (Devarajan et al. 2010; Patil et al. 2008). The effect of solvents on the shape of the nanoparticles has also been reported by other researchers (Peltonen et al. 2002). Scanning electron microscopy (SEM) images of the four different LIPOMER nanoparticles confirm irregular shape of iGMS LIPOMER nanoparticles and spherical/regular shape of glyceryl palmitostearate (sGPS) LIPOMER, stearic acid (sSA) LIPOMER and sGMS LIPOMER nanoparticles (Fig. 1). The iGMS LIPOMER nanoparticles did not reveal any defined geometric shape. Moreover, asperity was observed in iGMS LIPOMER nanoparticles hence, defined as asymmetric or irregularly shaped particles.

2.2. Protein patterns on LIPOMER nanoparticles

The obtained plasma protein adsorption patterns are displayed in Fig. 2. Qualitatively the patterns of all four formulations are nearly the same. That means on all samples similar proteins have adsorbed to the surface of the nanoparticles. The overall quantity of adsorbed proteins is very low, with only four different proteins identified on the adsorption patterns. The predominant groups of proteins are the apolipoproteins. Namely apolipoprotein A-I, A-IV, C-II and J have been identified. Furthermore, a quantitative overview of identified proteins is given in Fig. 3. Major differences in the relative amounts of adsorbed proteins on the surface of the four different particles can be seen. ApoA-I along with apoJ take 50.5% (of the total amount of detected proteins), exactly 25.4% and 25.1% on the surface of sGPS LIPOMER nanoparticles (Fig. 2, A). ApoC-II represents 9.3% and apoA-IV 2.6% of identified proteins on these nanoparticles. The quantitative protein adsorption results on the surface of sSA LIPOMER nanoparticles (Fig. 2, B) are different, as apoA-I is by far the dominant protein adsorbing to the surface with about 37.5%, followed by apoA-IV with 19.2%, apoC-II with 4.7% and apoJ 1.5%. Again, a complete diverse quantitative arrange-

Table: Particle size, polydispersity index (PDI) and entrapment efficiency of LIPOMER nanoparticles

Sample		Non-solvent phase	Particle size (mean ± SD) (n = 3)	PDI (mean ± SD) (n = 3)	% Entrapment efficiency (mean ± SD) (n = 3)
A	sGPS LIPOMER nanoparticles	50% v/v IPA	356.26 ± 2.81	0.101 ± 0.12	79.01 ± 2.61
B	sSA LIPOMER nanoparticles	50% v/v IPA	401.22 ± 3.61	0.196 ± 1.45	78.32 ± 0.35
C	sGMS LIPOMER nanoparticles	water	350.68 ± 5.85	0.185 ± 0.32	81.26 ± 1.62
D	iGMS LIPOMER nanoparticles	50% v/v IPA	394.21 ± 4.81	0.284 ± 0.61	75.29 ± 2.19

Nanoparticles were of spherical (s) or irregular (i) shape (GPS = glyceryl palmitostearate; SA = stearic acid; GMS = glyceryl monostearate; SD = standard deviation; IPA = isopropyl alcohol).

ment can be seen by looking at the adsorbed proteins on the surface of sGMS LIPOMER nanoparticles (Fig. 2, C). ApoC-II has adsorbed at the highest quantity with 23.8%, followed by apoA-I with 18.2%, apoJ with 2.9% and apoA-IV with 2.2%. On the surface of iGMS LIPOMER nanoparticles (Fig. 2, D) apoJ and apoA-I are present with the highest quantities, namely 19.7% and 19.1%. The amount of apoA-IV is 9.6% and apoC-II is 8.1%.

By comparing the four different identified plasma proteins, one can say that apoA-I has adsorbed on all four surfaces at least over 18%. ApoJ has been identified in high quantities (more than 19%) on sGPS and iGMS LIPOMER nanoparticles. ApoA-IV is highest on sSA LIPOMER nanoparticles (over 19%) and iGMS LIPOMER nanoparticles (over 9%) and apoC-II is dominant on sGMS LIPOMER nanoparticles (over 23%) (Fig. 3, lower). In summary, on each sample surface the same plasma proteins have adsorbed, but distinct quantitative differences can be remarked. A possible reason for the qualitatively similar patterns might be found by looking at the formulations, as all four samples consist of a lipid and an identical polymer (poly(vinyl methyl ether-co-maleic anhydride); PVMMA) as matrix material and 1% Tween 80. Apart from the particle shape of one sample, the other three formulations differ singularly in their lipid matrix. The diverse lipids used in the formulations are located inside the particles, covered by a surfactant, Tween 80. Müller and Heinemann (1989) postulated the concept of differential protein adsorption in the 1980's, saying that the protein adsorption on i.v. drug delivery systems depends on the physico-chemical properties of the i.v. drug carrier. Therefore, by presuming a similar surface it is just consequential that the resulting protein adsorption patterns are similar, too.

Tween 80 is a well investigated surfactant regarding protein adsorption and has already proven in previous experiments the characteristic of preferentially adsorbing apolipoproteins. In certain cases a noticeable amount of apoE could be identified resulting in a specific brain targeting potential (Göppert and Müller 2005b). In the present study no apoE has been identified on the surface of all four different nanoparticles. Thus, a potential brain targeting potential of the four formulations cannot be attested. Moreover, very interesting is the missing of albumin, which is by far the most present protein in human plasma (3500–5000 mg/dl). Also widely distributed in the human plasma are immunoglobulins (IgG: 700–1600 mg/dl) and fibrinogens (200–450 mg/dl). These two protein groups have

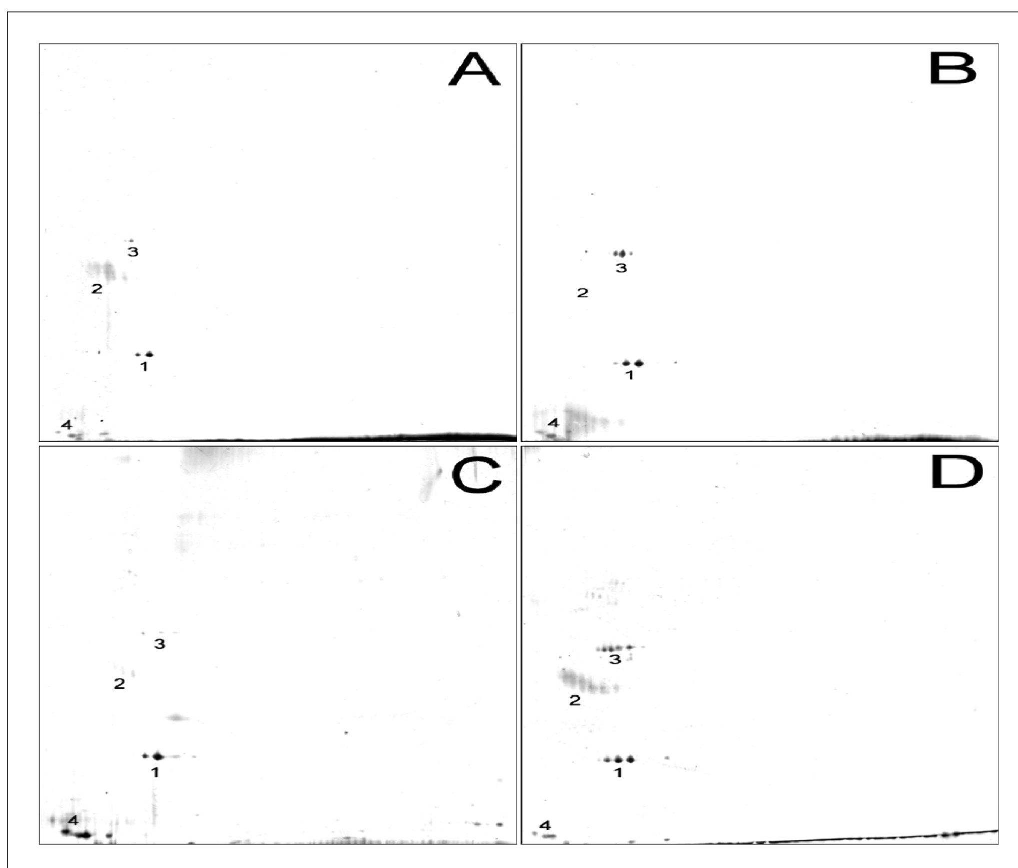

Fig. 2: Plasma protein adsorption patterns of sGPS LIPOMER nanoparticles (A), sSA LIPOMER nanoparticles (B), sGMS LIPOMER nanoparticles (C) and iGMS LIPOMER nanoparticles (D). Identified protein spots are: (1) ApoA-I, (2) apoJ, (3) apoA-IV and (4) apoC-II

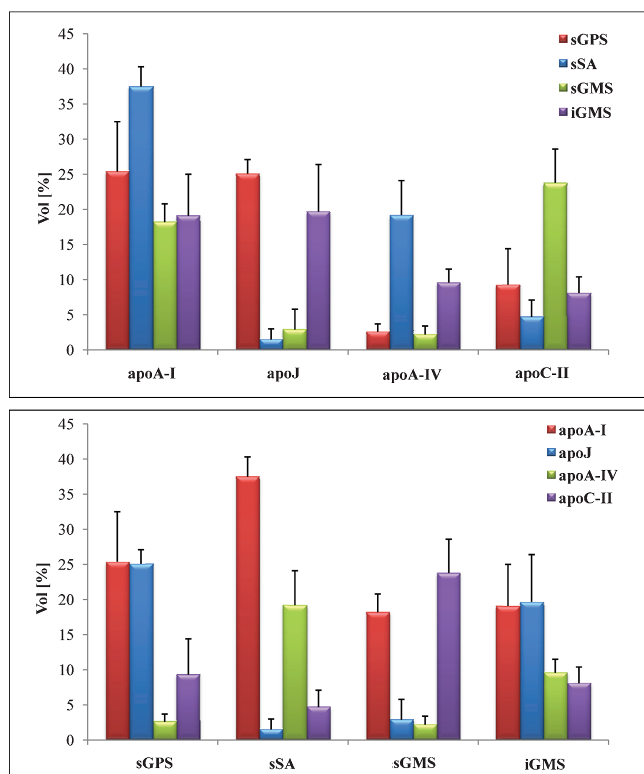

Fig. 3: Relative volume (in vol [%]) of the major proteins adsorbed on the surface of sGPS LIPOMER nanoparticles, sSA LIPOMER nanoparticles, sGMS LIPOMER nanoparticles and iGMS LIPOMER nanoparticles obtained from 2-D PAGE analysis (n = 3), grouped for each apolipoprotein (upper) and also per type of nanoparticles (lower), for reason of better comparison

surprisingly not been found on any particle surface either. A possible reason for that might be found in the Vroman effect. That means proteins being present in very high concentrations adsorb primarily on the surface of the particles and will be replaced by proteins with a higher affinity to the particle surface. This phenomenon takes place within a split second. Previous experiments have proven the existence of the Vroman effect on various particles, like polymeric particles (Blunk et al. 1996) or solid lipid nanoparticles (SLNs) (Göppert and Müller 2005a). Further studies have to evaluate, if there is a Vroman effect in the early phase of protein adsorption on LIPOMER nanoparticles or not.

With regard to the *in vivo* fate of the particles, the adsorbed plasma proteins are known to play a key role (Davis et al. 1993; Müller and Heinemann 1989). In the present study exclusively apolipoproteins have been identified on the particle surface. In detail, the proteins are apoA-I, apoA-IV, apoC-II and apoJ. A specific splenotropic targeting potential of one of these proteins has not been reported earlier in the literature. In most cases these apolipoproteins have been related to a transport across the blood brain barrier (BBB). ApoA-I and apoA-IV have shown a modulating effect on the transport across the BBB (Gessner et al. 2001). An additional adsorption of these two proteins reduced the hepatocyte uptake and enabled particles with adsorbed apoE the contact with receptors at the BBB. Recently, apo-I and apo-II have been reported to enhance phagocytic activity through ATP-binding cassette transporter (Tanaka et al. 2010). ApoC-II has been described to influence apoE mediated brain uptake, too, as it inhibits the binding of apoE to the receptor (Weisgraber et al. 1990). ApoJ is also able to cross the BBB (Elliott et al. 2010).

In a previous study different LIPOMER nanoparticles have been evaluated particularly with regard to enhanced splenic uptake (Patil et al. 2008). The biodistribution studies in rats suggested significant enhancement of iGMS LIPOMER nanoparticles in the spleen coincides with lower hepatic uptake. Fig. 4 gives an

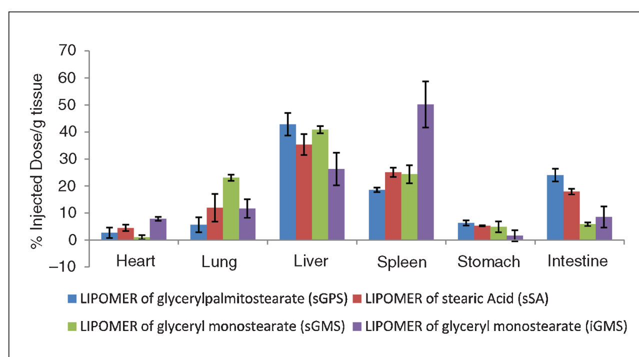

Fig. 4: Comparative biodistribution profile 5 h post dosing of different LIPOMER nanoparticles in Wistar rats (n = 4; mean ± standard deviation)

overview of comparative biodistribution 5 hours post dosing of the four different LIPOMER nanoparticles into rats. Further, an increased splenic uptake has been observed with increasing concentrations of GMS which was related to greater irregularity in shape. The impact of particle shape on phagocytosis and biodistribution of intravenously injected particles has been reported earlier, too (Champion and Mitragotri 2006, 2009; Decuzzi et al. 2010; Sharma et al. 2010).

The resulting protein adsorption patterns suggest similar *in vivo* behavior of all four LIPOMER nanoparticles based on qualitative similar protein adsorption. The fact, that a distinct different biodistribution in rats with a higher splenic and a lower liver uptake of iGMS LIPOMER nanoparticles has been obtained (Fig. 4), led to the implication of particle shape as determining parameter on bypassing liver macrophages and targeting to the spleen. The quantitative different arrangement of adsorbed proteins potentially takes up a super position on this effect.

2.3. *Phagocytosis*

Following i.v. administration, nanoparticles are mostly opsonised and phagocytosed by macrophages within seconds to a few minutes. Most phagocytic binding cannot occur without opsonization of the particles. Opsonization is performed by coating of particles with plasma proteins called opsonins, which namely include immunoglobulins, complementary factors, von Willebrand factor, thrombospondin, fibronectin, fibrinogen and mannose-binding protein (Moghimi et al. 2001). Additionally, opsonization is facilitated by the activation of complement system which comprises several circulating proteins like C1q and C3 fragments (Owens and Peppas 2006). Complementary systems are activated by the classical pathway, mannose-binding lectin pathway and the alternative pathway to generate a specific protease called C3 convertase (Tohyama and Yamamura 2006). IgG endorse phagocytosis by binding to the Fc receptor on phagocytic cells (Tanaka et al. 2010). Coating of particles with immunoglobulins, complement factors and fibrinogens promote phagocytosis and clearance of the particles from the blood stream while coating with albumin promotes circulation time of the particles by hindering phagocytosis (Shegokar et al. 2011). Adsorption of proteins on the particle surface dictates the *in vivo* fate of particles. For instance, *in vivo* adsorption of immunoglobulins on the particle surface led to rapid hepatic clearance (Nagayama et al. 2007) while adsorption of apolipoprotein E resulted in enhanced brain accumulation (Sun et al. 2004).

Once the particle becomes visible to macrophage cells after opsonization, macrophages phagocytize the particles. Phagocytosis is an actin dependent process and driven by the controlled rearrangement of the actin cytoskeleton (May and Machesky 2001). Large particles are phagocytized by the formation of actin

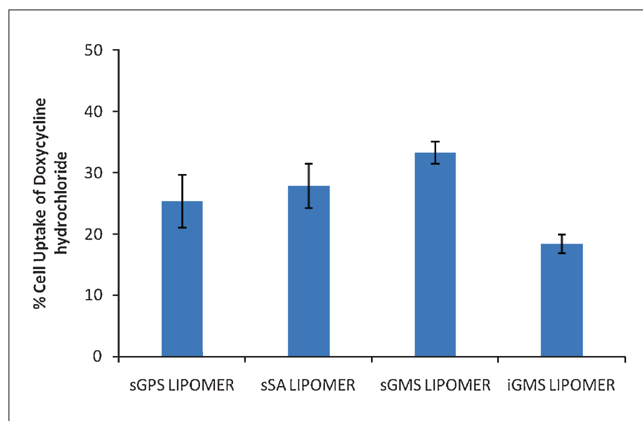

Fig. 5: Cell uptake (%) of DH from sGPS LIPOMER nanoparticles, sSA LIPOMER nanoparticles, sGMS LIPOMER nanoparticles and iGMS LIPOMER nanoparticles using human macrophage cell line U-937 of histocytic lymphoma organ (n = 3; mean ± standard deviation; mass balance of >90% was confirmed)

cups and rings around the particles. As the particles come in contact with the macrophage cell, it forms an actin cytoskeleton on the particle. Actin structure formation depends upon the local contact shape of the particle with the cell. In case of spherical particles, the cells form an actin cup and move on for complete internalization of the particle, while macrophage cells are not able to internalize non-spherical particles due to incomplete actin structure formation. Champion and Mitragotri (2006) proclaimed that the particle shape and not the particle size plays a dominant role in phagocytosis The authors used polystyrene particles of various size and shape and analyzed their engulfment. The conclusion for that has been found in particle shape, which influences initial phagocytosis during actin structure formation. Recently, the same authors published results of worm-like particles, whose shape inhibited the phagocytosis (Champion and Mitragotri 2009). The reason for this phenomenon can be found in a different contact angle for cell attachment. The results clearly demonstrate that changes in solely the parameter particle shape can lead to a completely different *in vivo* behavior.
Hence, while phagocytosis is influenced by shape of the nanoparticles we have not observed an effect of shape on opsonization. Whereas the adsorbed protein concentration varied, protein patterns on all the LIPOMER nanoparticles were qualitatively similar irrespective of chemical composition and shape. This finding corroborates the assumption, which has been proposed regarding accumulation of irregular nanoparticles in the spleen (Devarajan et al. 2010). It relates splenotropic behavior to the irregular shape and rigidity, analogous to recognition of defective red blood cells normally cleared by the spleen. The results observed suggest that the splenotropic behavior is independent of opsonization and rule out the possibility of adsorption of organ specific proteins in enhanced splenic uptake. It is therefore hypothesized that opsonization is independent of nanoparticles shape but could be related to the chemistry of the nanoparticles. However, the same was not apparent with the LIPOMER nanoparticles studied, as major qualitative differences in adsorbed proteins were not evident.

2.4. *In vitro cell uptake studies*

In vitro macrophage uptake studies revealed shape dependent internalization of LIPOMER nanoparticles. iGMS LIPOMER nanoparticles Exhibit 18 Exhibit 18% cell uptake, whereas in spite of similar composition sGMS LIPOMER nanoparticles possess 33% cell uptake after 1 h incubation (Fig. 5). These results are in agreement with the previous report on qualitative *in vitro* white blood cell (WBC) uptake studies wherein enhanced internalization of sGMS LIPOMER nanoparticles was observed as compared to iGMS LIPOMER nanoparticles (Devarajan et al. 2010). sSA and sGPS LIPOMER nanoparticles revealed 28% and 25% cell uptake respectively. These results confirm that iGMS LIPOMER nanoparticles were phagocytized less readily than the sGMS LIPOMER nanoparticles and approve the role of shape on phagocytosis as already mentioned earlier. Macrophages require creating actin structure for phagocytosis of particles. An actin structure could not be created on the surface of iGMS LIPOMER nanoparticles which could result in 1.8 fold decrease in internalization as compared to sGMS LIPOMER nanoparticles.

3. Experimental

3.1. *Preparation of LIPOMER nanoparticles*

LIPOMER nanoparticles were prepared by a modified nanoprecipitation method as reported previously (Devarajan et al. 2010; Patil et al. 2008). Briefly, PVMMA (200 mg), DH (100 mg), and the lipid (100 mg) were dissolved in 10 ml tetrahydrofuran (THF) and added drop-wise to 30 ml of a non solvent phase comprising of water and IPA (1:1) containing Tween 80 (1% w/v) under magnetic stirring. The lipids evaluated were GPS, SA and GMS. Magnesium acetate solution (3 ml; 5% w/v) was added to stabilize the nanoparticles and the dispersion allowed to stir until complete removal of solvent. GMS LIPOMER nanoparticles were prepared as described above, using water alone as the non-solvent phase. The LIPOMER nanoparticles were separated by centrifugation (28,360 × g, 30 min). The resultant pellet was redispersed in 5 ml distilled water using a probe sonicator (Vibronics ultrasonic processor P2, India, 20 KHz) for 5 min. The dispersions were lyophilized after addition of trehalose (8% w/v) as cryoprotectant. Particle size was determined by photon correlation spectroscopy using a Coulter N4 plus submicron particle size analyzer (Beckman Coulter, USA) at 25 °C. All measurements were taken at 90° scattering angle. The nanodispersions were diluted with water (filtered through 0.22 μm filter) and the particle size recorded. The nanoparticle dispersions were centrifuged (28,360 × g, 30 min) and the entrapment efficiency (%) was determined by analyzing DH in the supernatant with an UV-spectrophotometer (UV-1650PC, Shimadzu, Japan) at 275 nm. The % entrapment efficiency was calculated.

3.2. *Sample preparation for 2-D PAGE*

Freeze dried powder (50 mg) was redispersed in 1 ml Milli-Q® water by sonication using a bath sonicator (5 min). The samples have been incubated in human plasma (German Red Cross, Berlin, Germany; 37 °C, 5 min) and the ratio sample to plasma was 1:3 (0.3 ml to 1.2 ml) according to previous protein adsorption studies (Göppert and Müller 2004; Petri et al. 2007). The separation of the nanoparticles from the incubation medium by centrifugation has been described in detail previously (Harnisch and Müller 1998). Briefly, each sample has been centrifuged (23,000 × g, 1 h) in order to separate it from unbound plasma. Three subsequent washing processes, using a 0.05 M phosphate buffer pH 7.4 and 1 h of centrifugation have been performed to obtain a purified sample. Then a solubilizing solution containing of 10% (w/v) sodium dodecyl sulfate (SDS) and 2.3% (w/v) dithioerythritol (DTE) has been applied and heated up (95 °C, 5 min) to detach the adsorbed proteins from the sample surface (Cook and Retzinger 1992). A second solution, consisting of 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) DTE, 0.5% (w/v) tris (hydroxymethyl) aminomethane and 8 M urea, has been added to cover the previously used SDS, which would interfere with the separation in the first dimension (isoelectric focusing, IEF) of 2-D PAGE. The mixture has been stirred and centrifuged (23,000 × g, 15 min). Finally, 215 μl of the solution has been used as sample for the subsequent gel electrophoresis.

3.3. *Protein adsorption analysis by using 2-D PAGE*

The 2-D PAGE is a method that is used to separate proteins according to two different parameters, namely the isoelectric point (pI) and the size of the proteins. In the following study the 2-D PAGE has been performed using 18 cm IPG BlueStrips (Serva, Heidelberg, Germany) with a nonlinear immobilized pH gradient from 3 to 10 in the first dimension. The strips have been applied to the Multiphor II (GE Healthcare, Munich, Germany), which was equipped with the E752 power supply from Consort (Turnhout, Belgium). The second dimension was carried out in the Protean II xi multi-cell with a Power Pac 1000 (both from Bio-Rad Laboratories, Munich, Germany). Gels, having

a linear gradient from 8–16% acrylamide using BIS (N,N'-methylene-bis-acrylamide) as a crosslinker, were cast using the Protean II xi multi-gel casting chamber (18 × 18 × 1.5 mm) and the Model 495 gradient former (both from Bio-Rad Laboratories, Munich, Germany). Visualization of the gels, after completion of the second dimension, has been enabled using the Bio-Rad Silver Stain, derived from the method of Merril et al. (1981). Briefly, silver staining is a highly sensitive method based on the reduction of ionic to metallic silver. By following a defined protocol (Bio-Rad Silver Staining 2012) one receives most reliable results. Afterwards, digital images of the silver-stained gels were generated by using a high quality 12- to 16-bit grey scale scanner (UMAX PowerLook® IIII Image Scanner, Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins have been qualitatively identified by comparison of protein spots to online available reference maps (ExPASy/SWISS-2DPAGE, Swiss Institute of Bioinformatics, Geneva, Switzerland) (Gasteiger et al. 2003). Quantitative analysis of the identified proteins was accomplished by using the MELANIE III software from the Swiss Institute of Bioinformatics (Geneva, Switzerland). All other reagents used for 2-D PAGE analysis were analytical grade and bought from Serva (Heidelberg, Germany).

3.4. *In vitro cell uptake studies*

In vitro cell uptake studies were carried out using the human macrophage cell line U-937 of the histocytic lymphoma organ. iGMS, sGMS, sGPS and sSA LIPOMER nanoparticles equivalent to 10 μg/ml of DH were incubated with the cells at a cell density of 2×10^6 cells/ml, containing 10% fetal calf serum for 1 h at 37 °C in a 5% carbon dioxide atmosphere in a CO_2 incubator (NuAire, USA). After incubation, cells were separated from uninternalised nanoparticles by centrifugation (640 × g, 3 min). The supernatant was collected and the cells were lysed using 0.5% w/v SDS solution (200 μL). The DH content in the cell lysate and supernatant was determined using a HPLC (LC 900, JASCO International Co., USA). HPLC was performed on a Spherisorb® 250 × 4.6 mm HPLC cartridge prepacked with Spherisorb® 5 μm ODS2 (Waters, USA). The mobile phase comprised of methanol, water and acetonitrile in the ratio of 80:10:10 containing 0.1% v/v trichloroacetate and 0.02% ethylenediaminetetraacetic acid (EDTA). Chromatography was performed at room temperature under isocratic conditions at a flow rate of 1.0 mL/min. UV detection was carried out at a λ_{max} of 350 nm.

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