International Journal of Molecular Medicine and Advance Sciences, 1(2): 196-201, 2005

Surface Modified Solid Lipid Nanoparticles (SLN) Analysis of Plasma Protein Adsorption Patterns by two-dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

H. Weyhers, M. Lück, W. Mehnert, E.B. Souto and R.H. Müller
Department of Pharmaceutics, Biopharmaceutics and Biotechnology
Free University of Berlin Kelchstr. 31, D-12169 Berlin, Germany

Abstract: Solid Lipid Nanoparticles (SLN) are a biodegradable drug carrier system with the potential to be introduced to the clinic. Surface-modified SLN were produced and optimized using the hydrophilic block copolymers poloxamine 908 and poloxamer 407 as stabilizing agents. These two block copolymers are known to modify the organ distribution of colloidal non-biodegradable model carriers administered via the i.v. route when adsorbed on their surface (i.e. avoidance of Mononuclear Phagocytic System (MPS) recognition, i.e. liver/spleen uptake). The interactions of surface-modified SLN with blood proteins were studied by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The resulting protein adsorption patterns proved that all surface-modified SLN formulations showed an extraordinary low degree of interaction with the plasma proteins. A preferential adsorption of albumin, fibrinogen and apolipoproteins A-I, A-IV and C-III was detected. The presence of apolipoproteins A-I and A-IV is discussed as being responsible for a modulating effect on the organ distribution. From this data obtained up to now surface-modified SLN might be a biodegradable colloidal carrier system efficient in avoiding the MPS recognition.

Key words: 2-D PAGE, poloxamer 407, poloxamine 908, solid lipid nanoparticles

INTRODUCTION

Colloidal drug carriers with no special surface modification are generally taken up by macrophages of the Mononuclear Phagocytic System (MPS) after i.v. administration. This leads to their rapid elimination from the systemic circulation. The reason for the recognition by the MPS is the absorption of plasma components on the carrier's surface as soon as they enter the blood stream. This process known as opsonisation was found to accelerate the uptake by macrophages populations[1,2]. The qualitative composition of the adsorbed plasma proteins is a function of the drug carrier's surface characteristics[3,4]. The protein adsorption patterns of i.v. administered colloidal drug carriers are therefore regarded as a key factor for the subsequent organ distribution[2,5].

Several studies performed in the late eighties showed that the surface modification of hydrophobic polystyrene particles using hydrophilic block-copolymers from the poloxamer and poloxamine series led to changes in the body distribution. The presence of a hydrophilic polymer layer minimises the adsorption of opsonic factors and also reduces potential particle-cell interactions[2,6,7]. The modification of 60 nm Polystyrene (PS) particles with poloxamine 908 led to particles that were kept within the systemic circulation[8], while coating of 60 nm polystyrene particles with poloxamer 407 resulted in an accumulation of the carrier in the bone marrow[9], i.e. in endothelial lining cells[10] after i.v. administration. Coating with larger polystyrene model carriers (142 nm) with poloxamer 407 diverted them from the bone marrow. These particles circulated also in the blood like poloxamine 908-coated PS-particles[2].

The successes in avoiding liver and spleen uptake by applying a simple coating (adsorption) process have only been achieved with non-biodegradable polystyrene model particles but could not be transferred to biodegradable carrier systems for many years. The main reason for this is that the surfaces of biodegradable polymer nanoparticles are less hydrophobic compared to the polystyrene nanoparticles. The adsorption process of hydrophilic block-copolymers still takes place but because of the mores hydrophilic surfaces of biodegradable particles, thinner adsorption layers of block-copolymers are obtained[2]. As a result the thin adsorption layers of block copolymers are not efficient in preventing particles from the opsonisation process anymore.

Meanwhile, polyester particles with reduced uptake by MPS organs (i.e. liver, spleen) were produced by a precipitation process allowing sufficient coating[11]. However, these particles could not yet be produced on large scale.

Promising investigations were performed using fat emulsions surface modified with poloxamine 908[12]. These emulsions showed a prolonged blood circulation.

Corresponding Author: Tel: +49 30 83850678 Fax: +49 30 83850616

Uptake into liver and spleen macrophages was reduced to 30% *in vivo*. These results lead to the assumption that surface-modified nanoparticles, made from solid instead of liquid lipids, should circumvent or minimise the MPS recognition[13]. The development of the So-called Solid Lipid Nanoparticles (SLN) became an interesting alternative to the polymeric nanoparticles for the present purposes[14].

MATERIALS AND METHODS

Lipids, emulsifiers and reagents: Compritol (glycerol behenate) was a gift from Gattefossé (Weil, Germany), poloxamine 908 and poloxamer 407 were provided by the distributor Erbslöh GmbH (Düsselforg, Germany). The producer of the block copolymers was ICI (Middlesborough, GB). Lipoid S75 (soy lecithin) was a gift from Lipoid KG (Ludwigschaften, Germany). Human plasma was drawn from healthy male volunteers and stored at -30°C.

All chemicals used for 2D-PAGE were of analytical grade. Acrylamide, N,N,N,N''-Tetramethylethylenediamnie (TEMED), ammonium persulfate and Piperizine Diacylamide (PDA) were obtained from BioRad (Munich, Germany). Nonidet-P 40, Tris and Cholamido propyldimethylhy droxy propan esulfonate (CHAPS) were from Sigma Chemicals (Deisenhofen, Germany). Carrier ampholytes pH of 3.5-10 and 4-8 were from BDH (Poole, UK), pH 8-10.5 from Pharmacia LKB (Uppsala, Sweden). All other chemicals according to Hochstrasser[15] were either from Fluka Chemie AG (Buchs, Switzerland) or from Merck (Darmstadt, Germany).

Preparation of SLN: SLN were prepared according to Weyhers[16] by adding water of 80°C containing the emulsifier to the melted lipid. The mixture was stirred using an Ultra Turrax T25 (Janke and Kunkel GmbH & Co KG, Staufen, Germany) for 1 min at 10 000 rpm. The pre-emulsion was homogenized using a Labsonic 2000 ultrasonic probe (Braun Melsungen AG, Melsungen, Germany). The power output was 200 or 300 W for 2 or 3 min, respectively. For details see (Table 1).

Table 1: Composition and production parameters of prepared SLN batches using an ultrasonic probe (surface modifying agents: 407 -Poloxamer 407, 908-Poloxamine 908, S75-Lipoid S75)

System	407	908	407/S75	908/S75
Content of lipid (%)	5.0	5.0	5.0	5.0
Content of emulsifier (%)	2.0	2.5	2.2/0.3	2.2/0.3
Production parameters				
Power output(W)	110	110	200	300
Sonication time(min)	2	2	2	3

Table 2: Particle size characterisation of surface-modified SLN by Photon Correlation Spectroscopy (PCS) and Lased Diffraction particle size analysis (LD)

			LD volume distribution		
System	PCS diameter	PI	D90%	D95%	D99%
407	248 nm	0.224	0.69 μm	0.84 μm	1.35 μm
908	272 nm	0.201	0.81 μm	0.99 μm	1.57 μm
407/S75	158 nm	0.132	0.51 μm	0.60 μm	0.85 μm
908/S75	174 nm	0.213	0.62 μm	0.73 μm	1.00 μm

Characterization of SLN: Particle size was determined by Photon Correlation Spectroscopy (PCS) using the Malvern Zetasizer IV (Malvern Instruments, UK) and by laser diffractometry (LD) using the Coulter LS 230 (Coulter Electronics, Krefeld, Germany). PCS data are listed as follows: average mean of the particles (PCS diameter) and polydispersity index (PI). LD analysis is based on the particle's volume distribution. The selected parameters were the D99, D95 and D90%, which mean that e.g. 90% of the measured particles (volume distribution) are smaller than the values listed [μm]. These LD parameters were found to be sensitive for the determination of particles aggregates. (Table 2).

Sample preparation and 2-D PAGE: A volume of 300 il of each surface-modified SLN dispersion (5% m/m) was incubated in 2200 μl undiluted citrate stabilised human plasma at 37°C for 5 min. Particles were separated from plasma by using a centrifugation step. Satisfying separation results were obtained by adding glucose to raise the density of plasma. The content of glucose was limited to 3% m/m to minimise possible osmotic effects like changes in plasma protein conformation or protein desorption differed neither qualitatively nor quantitatively using this alternative separation technique. 2-D PAGE was performed as described previously[6,15]. In the first dimension, the proteins were separated according to their Isoelectric Point (pI) (isoelectric focussing). Isoelectric focussing was performed in a model 175 tube cell from Bio-Rad (Munich, Germany). For isoelectric focussing carrier ampholytes were used. In the second dimension, the separation was carried out according to the Molecular Weight (MW) of the proteins (SDS-PAGE). For SDS-PAGE the Bio-Rad Protan II 2-D multi-cell, the multi-gel casting chamber (160x160x1.5 mm gels) and model 395 gradient former were used. The first and the second-dimensional separation were performed with the Bio-Rad 1000/500 power supply. After SDS-PAGE the gels were silver stained. The silver-stained gels were scanned using a laser densitometer (Personal Densiometer, Molecular Dynamics, Krefeld, Germany). The protein adsorption patterns were analysed using the MELANIE software[17]. The amount of protein adsorbed was assessed in a semi-quantitative manner on the basis o f spot size and

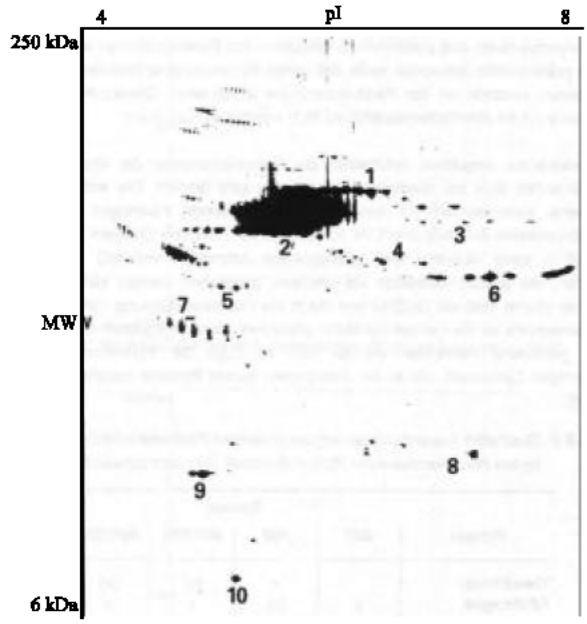

Fig. 1: 2-D PAGE gel of human plasma (1 = Transferring, 2 = albumin, 3 = C3b, 4 = Fibrinogen á, 5 = Fibrinogen Â, 6 = Fibrinogen Ã, 7 = Iggã, 8 = Apolipoprotein A-iv, 9 = Haptoglobolin Â Chain, 10 = Ig Light Chains, 11 = Apolipoprotein A-i, 12 = Transthyretin)

intensity of silver staining. For further details refer to Blunk[5,6]. A 2-D PAGE gel of human plasma is exemplary (Fig. 1).

RESULTS AND DISCUSSION

The plasma protein adsorption patterns of the analysed SLN batches are shown in Fig. 2a-d. The complete gels are shown (pI 4.0-8.0 [from left to right, non-linear], MW 6-250 kDa [from bottom to top, non-linear]). All surface-modified SLN formulations showed only a low degree of interaction with the plasma proteins. For a better assessment of the results a 2-D PAGE gel human plasma is shown in Figure 1. Some of the main proteins are marked and listed.

Blunk determined a preferential adsorption of plasma proteins on polystyrene model carriers. Preferential adsorption means that some plasma proteins adsorbed in a minor extent others were enriched onto the particle surface compared to the bulk plasma[6]. This phenomenon was also observed for surface-modified SLN.

When studying the gels, the protein adsorption patterns seemed to be similar in a qualitative manner. The main adsorbed proteins were albumin, fibrinogen and the apolipoproteins A-I, A-IV and C-III. Regarding the 407 and the 908/S75 formulations an adsorption of IgG to a very low extent could be observed. Blunk assumes that the major proportion of the IgG adsorption takes place during the washing steps in the separation process of the particles from plasma[6]. It is discussed that this phenomenon, which was exclusively observed for the IgG, is a result of changes in the IgG solubility taking place during the dilution of plasma. Therefore, the IgG adsorption on nanoparticles is regarded as being artificial in most cases[6].

For the 908, 407/S75 and 908/S75 formulations a haemoglobin spot was detected. For the 407, 407/S75 and 908/S75 formulations a spot was observed that could not be identified using the plasma map according to Hughes *et al.*[18] and to Golaz *et al.*[19] (Table 3).

The quantitative evaluation of the gels expresses again the extremely low interaction of plasma proteins with the surface-modified SLN. The amount of adsorbed proteins are listed in cpm (counts per minute): cpm is an arbitrary unit of the MELANIE software. For all gels very low cpm values in the range of 63 to 225 were determined (Table 4). One of the main reasons for a low degree of protein interaction with particle surfaces was found to be a result of the surface density of the adsorbed block-copolymer[20]. Furthermore, the chain length of

Table 3: Qualitative evaluation of adsorbed plasma proteins on surface-modified SLN (x-protein detected, (x)-protein detected but weak spots)

	System			
Protein	407	908	407/S75	908/S75
Transferring		x	(x)	(x)
Fibrinogen	x	(x)	x	x
Albumin	x	x	x	x
Iggγ	x			x
Ig Light Chains				x
Apo A-I	x	x	x	x
Apo A-Iv	x	x	x	x
Apo C-III		x	x	x
Apo J		(x)		
Haemoglobin		x	x	x
Non identified spots	1		1	1

Table 4: Quantitative evaluation of adsorbed plasma proteins (absolute amounts in cpm ; proteins in % of total amount)

System	407	908	407/S75	908/S75
Counts [cpm]	63	107	114	225
Protein [% cpm]				
Transferring	0	2.99	0.75	0.40
Fibrinogen (α,β,γ)	12.14	1.19	27.78	17.35
albumin	41.74	58.78	28.69	13.73
IgG	3.77	0	0	1.89
apo A-I	14.04	10.33	10.74	20.98
apo A-IV	19.83	12.49	13.23	16.34
apo J	0	0.53	0	0
apo C-III	0	6.46	2.57	17.65
Haemoglobin	0	3.64	7.62	3.25

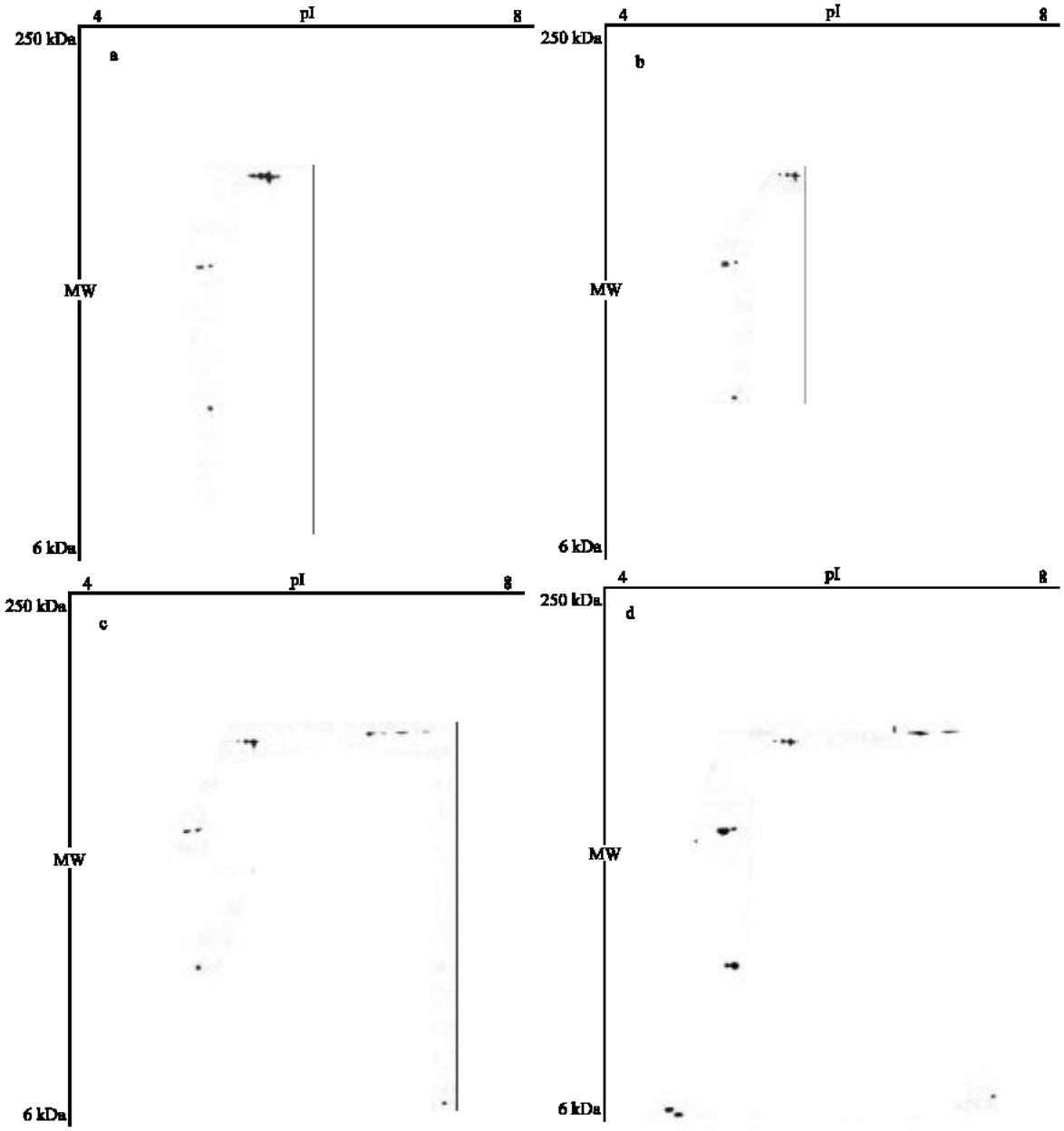

Fig. 2: Plasma protein adsorption patterns of surface modified SLN. (a) Pattern of poloxamer 407 modified SLN; (b) Pattern of poloxamine 908 modified SLN; (c) Pattern of poloxamer 407/Lipoid S75 modified SLN; (d) Pattern of poloxamine 908/Lipoid S75 modified SLN

the polyethylene glycol moiety of the block- copolymer seems to be very important[6,7]. In view of the results presented above, a high surface density of PEO-chains can be postulated when surface modified SLN are prepared using poloxamine 908 and poloxamer 407, respectively, as modifying agents. In addition, a windscreen-wiper effect of hydrated PEO-chains is discussed to support a minor tendency of interaction with plasma proteins[21].

Albumin was found to be the dominant protein on the SLN gels in a range from 13.7 to 58.8% of the overall detected proteins [% cpm] (Table 4). Surprisingly, the amount of adsorbed albumin was decreased for the lecithin containing formulations (908/S75: 13.7%; 407/S75:

28.7%) compared to the 908 and 407 formulations (908: 58.8%; 407: 41.7%).

When analysing the apolipoprotein A-I (apo A-I) and A-IV (apo A-IV) spots the relative dimension of adsorption was found to be remarkable. For apo A-I adsorbed amounts in the range of 10.3% to 20.9% were detected, for apo A-IV adsorbed amounts from 12.9% to 19.8% were found. Blunk showed for unmodified polystyrene particles that these proteins only play a minor role when looking at the adsorbed plasma proteins (apo A-I: 0.6%, apo A-IV 0.2%)[6]. For surface-modified SLN the relative weighting of the apolipoproteins mentioned above increases.

It was shown that apo A-I and apo A-IV counteracted the enhanced receptor-mediated uptake of apo E-coated liposomes by liver cells[22]. Apo A-I and apo A-IV were shown to displace apo E from phosphatidylcholine liposomes. Thus, it was proposed that apo A-I and apo A-IV have a modulating influence on the *in vivo* uptake of i.v. administered colloidal drug carriers[23].

CONCLUSIONS

For all surface-modified SLN formulations studied a distinctly low plasma protein adsorption was detected. A low degree of plasma protein interactions with particle surfaces is generally due to a trick adsorption layer in conjunction with a sufficient surface-density of adsorbed block-copolymers[2,6,7]. Furthermore, a windscreen-wiper effect of hydrated PEO-chains is discussed to prevent substantial interactions of plasma proteins with the hydrophilized particle surfaces[21].

Considering the low amount of plasma protein interaction with the modified SLLN surfaces it might be possible to circumvent phagocytosis by macrophages of MPS. This might result in a prolonged systemic circulation of the surface-modified SLN formulations. At this point ; without a complete knowledge of the correlation between the adsorption pattern and the organ distribution; no conclusive prediction can be made about the *in vivo* distribution. The effects of the plasma protein adsorption including the preferential adsorption of apo A-I and apo A-IV on the organ distribution have to be studied *in vivo*. Theses studies will have to show if the preferential adsorption of apo A-I and apo A-IV can be regarded as a key factor for a prolonged blood circulation.

REFERENCES

1. van Oss, C.J., D.R. Absolom and A.W. Neumann, 1983. Interaction of phagocytes with other blood cells and with pathogenic and nonpathogenic microbes. Ann. N Y. Acad. Sci., 416: 332-350.
2. Müller, R.H., 1991. Colloidal Carriers for Controlled Drug Delivery and targeting. Modification, characterisation and *in vivo* distribution, (Ed. W.V. Stuttgart).Boca Raton: CRC Press.
3. Müller, R.H. and S. Heinemann, 1989. Surface Modelling of Microparticles as Parenteral Systems with High Tissue Affinity. In: Bioadhesion - Possibilities and Future Trends, Gurny, R., H.E. Junginger, (Eds) 1989, Wissenschaftliche Verlagsgesellschaft: Stuttgart, Germany. pp: 202-214.
4. Juliano, R.L., 1988. Factors affecting the clearence kinetics and tissue distribution of liposomes, microspheres and emulsions. Adv. Drug Del. Rev., 2: 31-54.
5. Blunk, T., D.F. Hochstrasser, J.C. Sanchez, B.W. Müller and R.H. Müller, 1993. Colloidal carriers for intravenous drug targeting: Plasma protein adsorption patterns on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis, 14: 1382-1387.
6. Blunk, T., 1994. Plasma protein adsorption auf kolloidalen arzneistoffträgern - analytik, korrelation mit oberflächeneigenschaften, implikationen für das drug targeting. In: Ph.D Thesis., University of Kiel: Kiel.
7. Davis, S.S., L. Illum, S.M. Moghimi , M.C. Davies and C. *et al.*, 1993. Microspheres for targeting drugs to specific body sites. J. Control. Release, 24: 157-163.
8. Illum, L., S.S. Davis, R.H. Müller, E. Mak and P. West, 1987. The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilized with a block copolymer-poloxamine 908. Life Sci., 40: 367-374.
9. Illum, L. and S.S. Davis, 1987. Targeting of colloidal particles to the bone marrow. Life Sci., 40: 1553-60.
10. Porter, C.J., S.M. Moghimi, L. Illum and S.S. Davis, 1992. The polyoxyethylene/polyoxypropylene block co-polymer poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow. FEBS Lett.,305: 62-66.
11. Dunn, S.E., S. Stolnik, M.C. Garnett and M.C. Davies *et al* ., 1994. Biodistribution studies investigating poly(lactide-co-glycolide) nanospheres surface modified by novel biodegradable copolymers. In: Proc. Intl. Symp. Control. Rel. Bioact. Mater.
12. Illum, L., P. West, P. Washington and S.S. Davis, 1989. The effect of stabilising agents on the organs distribution of lipid emulsions. Intl. J. Pharm.., 54: 41-49.

13. Müller, R.H., K. Mäder and S. Gohla, 2000. Solid Lipid Nanoparticles (SLN) for controlled drug delivery : A review of the state of art. Eur. J. Pharm. Biopharm., 50: 161-177.
14. Müller, R.H. and J.S. Lucks, 1991.Arzneistoffträger aus festen Lipidteilchen, Feste Lipidnanosphären (SLN).
15. Hochstrasser, D.F., M.G. Harrington, A.C. Hochstrasser, M.J. Miller and C.R. Merril, 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. Anal. Biochem., 173: 424-435.
16. Weyhers, H., 1995. Feste Lipid Nanopartikel (SLN) für die gewebsspezifische arzneistoffapplikation, herstellung, charakterisierung oberflächenmodifizierter formulierungen, in Ph.D Thesis. Freie Universität Berlin: Berlin.
17. Appel, R.D., D.F. Hochstrasser, M. Funk, J.R. Vargas, C. Pellegrini, A.F. Muller and J.R. Scherrer, 1991. The MELANIE project: From a biopsy to automatic protein map interaction by computer. Electrophoresis, 12: 722-735.
18. Hughes, G.I., S. Frutiger, N. Paquet, F. Ravier and C. Pasquali *et al*., 1992. Plasma protein map: An update my microsequencing. Electrophoresis, 13: 707-714.
19. Golaz, O., G.J. Hughes, S. Frutiger, N. Paquet and A. Bairoch *et al*., 1993. Plasma and red blood cell protein maps: Update1993. Electrophoresis, 14: 1223-1231.
20. Andrade, A.J., 1985. Protein Adsorption, Ed. Andrade, A.J.E. (Ed.). New York: Plenum Press. 1-89.
21. Nagaoka, S., Y. Mori, H. Tanzanawa and S. Nishiumu, 1984. Interaction between Blood Components and Hydrogels with Poly(oxyethylene) Chains. In: Polymers as Biomaterials, Shalaby, S.W., A.S. Hoffmann, (Eds.). Plenum Press: New York. pp: 361-374.
22. Bisgaier, C.L., M.V. Siebenkas and K.J. Williams, 1989. Effects of apolipoproteins A-IV and A-I on the uptake of phospholipid liposomes by hepatocytes. J. Biol. Chem., 264: 862-866.
23. Scherphof, G.L., 1991. *In vivo* behaviour of liposomes: Interactions with the Mononuclear Phagocyte System and Implications for Drug Targeting. In Handbook of Experimental Pharmacology, Juliano, R.L.E., (Ed.). Springer Verlag: Berlin, Heidelberg. pp: 285-327.