ELSEVIER

International Journal of Pharmaceutics 144 (1996) 115–121

international journal of pharmaceutics

Biodegradation of solid lipid nanoparticles as a function of lipase incubation time

Rainer H. Müller*, Dörte Rühl, Stephan A. Runge

Department of Pharmaceutics, Biopharmaceutics and Biotechnology, The Free University of Berlin, Kelchstraße 31, D-12169 Berlin, Germany

Received 25 July 1996; accepted 4 September 1996

Abstract

The in vitro degradation of solid lipid nanoparticles (SLN) was studied in solutions of pancreatic lipase/colipase. Degradation was followed by turbidity measurements of SLN suspensions and by the determination of the formed free fatty acids (FFA). The degradation velocity was found to depend on the nature of the lipid matrix, being highest for Dynasan 114, medium for cetylpalmitate and relatively slow for lipids with longer fatty acid chains (Compritol ATO 888). The surfactant used for SLN stabilization had a dominating effect on degradation velocity. Use of the sterically stabilizing poloxamer 188 could prevent the in vitro degradation of well-degradable Dynasan 114 particles. This was attributed to the lack of anchoring of the lipase to the particle surface-prerequisite for enzymatic degradation. The dominating effect of the surfactant can be exploited to design SLN with optimum degradation velocity and matrix-controlled drug release-independent on the nature of the lipid matrix.

Keywords: Biodegradation; Colipase; Free fatty acids; Lipase; Solid lipid nanoparticles; Surfactant

1. Introduction

Solid lipid nanoparticles (SLN) are an alternative carrier system to polymeric nanoparticles with increasing attention from different research groups (Müller and Lucks, 1991; Schwarz et al., 1993; Müller et al., 1995; Siekmann and Westesen, 1992; Boltri et al., 1995; Gasco and Morel, 1990; Amselem et al., 1992). SLN combine advantages of polymeric nanoparticles (solid matrix for controlled release), emulsions and liposomes (physiological material of high toxicological acceptance, facility of industrial scale production by high pressure homogenization). The areas of application are very broad. The SLN can be incorporated in topical or ophthalmic formulations or administered perorally for controlled release inside the gastrointestinal tract (GIT). Their matrix

* Corresponding author. Tel.: +49 30 77000416; fax: +49 30 77000416.

PII S0378-5173(96)04731-X

can protect drugs against chemical degradation, the general adhesive properties of small particles to the gut wall opens the perspectives of less variable and/or enhanced bioavailibility of drugs. Intravenous administration of SLN can be used for the delivery of poorly water-soluble drugs avoiding toxicologically less acceptable solubilizing excipients (e.g. Cremophor EL) or in general for drug targeting similar to polymeric nanoparticles.

The degradation velocity of particles affects the toxicological acceptance (e.g. concentration of degradation products) and also the matrix-controlled release of drugs. The knowledge of the degradation velocity and the effects of the excipients used (nature of lipid and surfactant) are essential prerequisites for the controlled design of SLN with optimized in vivo performance. Injection of liquid oils is commonly used (e.g. parenteral nutrition) and many in vitro and in vivo degradation data are available, however little is known about injected solid lipids (Weyhers, 1995; Weyhers et al., 1995; Weyhers et al., 1996). Solid lipids are used as powders in tabletting, however the size of SLN is much smaller and the surface is stabilized by surfactants affecting possibly the degradation velocity. In this study, turbidity measurements of nanoparticle suspensions (Müller et al., 1990, 1992; Wallis and Müller, 1993) and determination of free fatty acid formation were investigated with regard to their suitability to follow SLN degradation. The degradation was studied as function of the nature of the lipid matrix and the surfactants used for particle stabilization, sodium cholate and poloxamer 188.

2. Materials and methods

2.1. SLN production and composition

The SLN used were produced by high pressure homogenization as described previously (1000 bar, three homogenization cycles) (Müller et al., 1995). Briefly, the melted lipid (2 g) was added to 38 g surfactant containing distilled water at 70°C (resulting dispersion: 5% lipid, 1% surfactant). This mixture formed a pre-emulsion after stirring for 1 min with an Ultra-Turrax K18 at 9500 rpm (Jahnke and Kunkel GmbH und Co KG, Staufen, Germany).

As lipids were used Dynasan 114 (triglyceride of myristic acid) provided by Hüls AG (Witten, Germany), Compritol ATO 888 (glycerolbehenate) as a gift from Gattefossé (Weil am Rhein, Germany) and cetylpalmitate purchased from Caelo (Germany). Sodium cholate from Sigma-Aldrich GmbH (Deisenhofen, Germany) and poloxamer 188 (Pluronic F68) as a gift from BASF AG (Ludwigshafen, Germany) were used as surfactants. Degradation experiments were performed after a storage period of 7 days at 4°C in order to ensure recrystallization of the lipid. Degree of crystallinity was assessed by differential scanning calorimetry (DSC).

2.2. Enzymes for SLN degradation

Purified lipase from porcine pancreas, type IV-S (30 000 U/mg solid) and colipase from porcine pancreas (lyophilized powders, essentially salt free) was obtained from Sigma-Aldrich GmbH. One unit lipase will hydrolyse 1.0 μ equivalent of fatty acid from olive oil in 1 h at pH 7.7 at 37°C. Lipase from porcine pancreas is very similar to that of human pancreas lipase. Both display the same molecular weight, the same composition of amino acids and a similar reactivity in the sulphide groups (Borgström, 1975).

2.3. Borate/boric acid buffer

The borate/boric acid buffer of the Gennan Pharmacopoeia was slightly modified by replacing the sodium chloride with calcium chloride. The buffer consisted of 2.85 g sodium tetraborate, 10.5 g boric acid and 2.5 g calcium chloride dissolved in 1000 ml Aqua dest. Calcium was added because it acts as activator of the lipase. The amount of $CaCl_2$ used in the buffer is below the 5 mM used by Sigma in the enzymatic assay of lipase. It needs to be reduced because it leads to flocculation of the SLN (zur Mühlen, 1996).

2.4. Enzymatic assay

The lyophilized lipase and colipase were dissolved in distilled water, respectively. One milliliter of lipase solution (2000 U/ml) was mixed with 0.6 ml colipase solution (50 μg colipase/ml) resulting in final concentrations of 2000 U/ml lipase and 30 μg colipase in 1.6 ml water (i.e. a relation colipase to lipase of 15 μg/1000 units). During an incubation time of 15 min at 37°C the lipase/colipase complex, necessary for absorption on the lipid particle surface was formed. To this pre-incubated mixture of 1.6 ml, a volume of 380 μl 0.01 M borate/boric acid buffer and 20 μl SLN dispersion (5% lipid, 1% surfactant in distilled water) were added yielding the dispersion for the degradation test. The buffer is used to keep the pH in the range of the activity maximum of the pancreatic lipase (between pH 7 and 9) and to buffer the formed free fatty acids. This assay was previously optimised by zur Mühlen (zur Mühlen, 1996).

2.5. Degradation tests

The nanoparticle dispersion was incubated at 37°C in an incubator. The maximum hydrolysis speed for lipase is observed at a temperature of 39°C. At 37°C, the speed is still very close to this maximum value (Keller et al., 1992). Samples of 50 μl (turbidity assay) and of 10 μl (colour assay) were drawn at certain times between 1 and 60 min and investigated in parallel by photometry (turbidity assay) and by determination of the formed free fatty acids using a colour assay. As controls, SLN in enzyme-free buffered solution were investigated.

Particle degradation can be followed by turbidity measurements, that means the decrease in absorption due to particle size reduction as a function of time, for details (Müller et al., 1990, 1992). Absorption was measured at 350 nm, the lipase/colipase displayed no signs of absorption in this range. The measured absorption was normalized to 1 for reasons of better comparison, measurements were performed using a Uvikon 940 (Kontron Instruments, Neufahrn, Germany). In parallel, the size of the SLN was determined by photon correlation spectroscopy (PCS-N4Plus Coulter corporation, Miami, USA). The initial sizes and polydispersity indices of the particles were 156 nm/0.14 (Dynasan 114, sodium cholate), 148 nm/0.24 (Compritol ATO 888, sodium cholate), 121 nm/0.13 (cetylpalmitate, sodium cholate) and 198 nm/0.22 (Dynasan, poloxamer 188), respectively.

The determination of the free fatty acids is carried out using the NEFA C Testkit (Wako Chemicals GmbH, Neuss, Germany). This is an enzymatic in vitro colour test which quantitatively determines the non-esterified fatty acids in serum and plasma. Non-esterified fatty acids (NEFA) in medium, when treated with acyl-CoA synthetase (ACS) in the presence of adenosine triphosphate (ATP), magnesium cations and CoA-SH, form the thiol ester of CoA (acyl-CoA), and the by-products adenosine monophosphate (AMP) and pyrophosphate (PPi). In the following part of the procedure, the acyl-CoA is oxidized by adding acyl-CoA oxidase (ACOD) to produce hydrogen peroxide which in the presence of added peroxidase (POD) allows the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)aniline (MEHA) with 4-aminophenazon to form a purple-colored adduct with an adsorption maximum at 550 nm:

$$\text{R-COOH} + \text{ATP} + \text{CoA-SH} \xrightarrow{\text{ACS}} \text{Acyl-CoA} + \text{AMP} + \text{Ppi}$$

$$\text{Acyl-CoA} + \text{O}_2 \xrightarrow{\text{ACOD}} \text{2,3-trans-Enoyl-CoA} + \text{H}_2\text{O}_2$$

$$2\text{H}_2\text{O}_2 + \text{4-aminophenazon} + \text{MEHA} \xrightarrow{\text{POD}} \text{Chinoimin-colouring} + 4\text{H}_2\text{O}$$

The intensity of the red colouring is proportional to the concentration of the non-esterified fatty acids in the test. The extinction maximum is 550 nm (Wako Chemicals, 1993).The extinction was measured by an Easy Rider EAR 400 AT from SLT-Labinstruments, Grödig, Austria.

3. Results and discussion

The SLN were produced with the bile salt sodium cholate because it is a physiological surfactant of the gastrointestinal tract playing a role on fat digestion. In addition, it yielded dispersions with high physical stability minimizing particle aggregation in enzymatic solution (see below). Particle degradation was performed in a pancreatic lipase/colipase solution because of the presence of bile salts in the particle surface. Bile salts inactivate lipase if colipase is not present. The protein colipase is a co-factor of pancreatic lipase which anchors the lipase onto the triglyceride surface, despite the presence of bile salts (Hofmann, 1978). In small concentrations, bile salts promote the necessary adsorption of the lipase on the surface of the lipid particles. At bile salt concentrations above 0.2 mM, the lipase quickly loses its ability to adsorb, this ability is practically lost above 1.5 mM bile salt (Lairon et al., 1978). Here, the bile salts wash the lipase off the surface of the particle. In contrast, the anchoring of the lipase/colipase complex onto the surface is stable enough to remain active also at bile salt concentrations above 20 mM (Lairon et al., 1978; Wollesen, 1981; Momsen and Brockman, 1976). As a further lipase activation mechanism, a buffer containing calcium instead of sodium chloride is used. In the absence of calcium in the incubation medium, the activity of the lipase with the addition of colipase and bile salts is more than 90% below that which is obtained with calcium (Lairon et al., 1980).

Following the particle degradation by turbidity measurements showed a distinct, almost exponential decrease within 60 min of incubation indicating a fast degradation of the particles (Fig. 1).

The curve flattened at about 20% absorption when approaching 60 min incubation time. Parallel PCS measurements showed an increase in particle size from 156 to 383 nm after 1 h incubation. The degrading particles aggregated, probably due to the loss of stabilizing surfactant during the degradation process. At 60 min the decrease in absorption due to particle degradation is compensated by an increase in absorption due to the larger size of the particle aggregates, a phenomenon described in more detail previously (Müller et al., 1990). In addition, the lipase starts to lose activity after approximately 60 min.

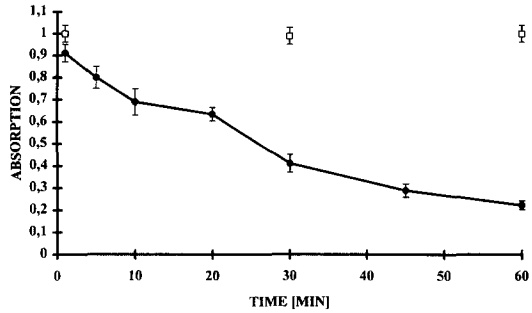

Fig. 1. Turbidity assay: degradation of SLN (Dynasan 5%, sodium cholate 1%) as a function of incubation time in a lipase/colipase buffer medium (●) and as control in enzyme-free buffer medium (□) (normation of absorption to 1).

The turbidity assay is fast and easy to perform, essential prerequisites for a broader screening of the degradation as function of SLN composition and the enzymes present in the environment. The determination of the free fatty acids (FFA) by a kit is distinctly more expensive. However, it allows to follow the degradation on the molecular level and avoids interference with aggregation of partially degraded particles. A distinct increase in FFA was observed for the Dynasan SLN during the first 30 min, approaching a maximum value at 60 min incubation (Fig. 2). This kinetics of FFA formation corresponds well with the turbidity

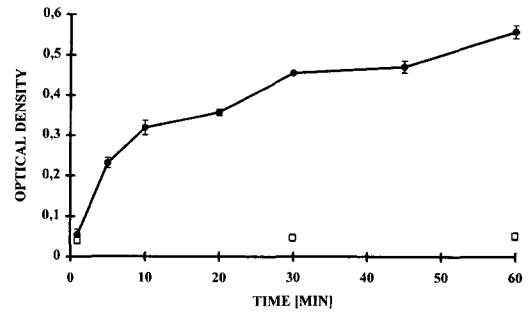

Fig. 2. The increase of free fatty acids (FFA) during the incubation of SLN from Fig. 1 (Dynasan 5%, sodium cholate 1%) in a lipase/colipase, buffer medium (●) as a function of time. As control, the incubation of SLN in enzyme-free buffer (□) was monitored. The concentration of FFA is expressed as optical density (OD) in the colour test.

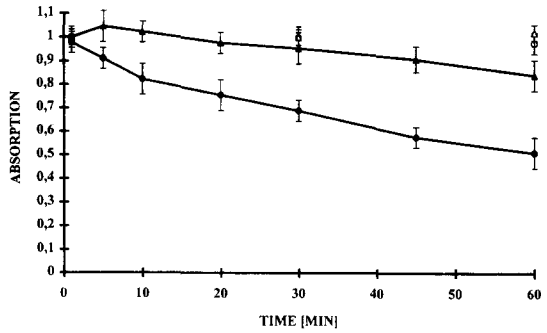

Fig. 3. Turbidity assay: degradation of Cetylpalmitate (●) and Compritol SLN (▲) as a function of incubation time in a lipase/colipase buffer medium and as control in enzyme-free buffer medium (o, Δ) (normation of absorption to 1).

data (Fig. 1) indicating the suitability of this assay for degradation studies.

Lipase hydrolyses exclusively the external fatty acid chains of the triglycerides without differentiating between position 1 and 3 (Hofmann and Borgström, 1962). The fatty acid chains on position 2 are only hydrolysed if they are intramolecularly moved to the outer position. At a given FFA formation, a maximum amount of triglyceride is degraded if only diglycerides are formed, less if monoglycerides are built, less of the triglyceride is degraded if degradation continues to glycerol. On the basis of the FFA concentration measured after 60 min incubation, the theoretical percentage of degraded triglyceride was calculated assuming the degradation to monoglycerides and alternatively to glycerol. For the sodium cholate-stabilized Dynasan SLN the data were 100% and 68%, respectively. The pronounced degradation of the Dynasan particles can be explained by the chemical structure of the lipid. Lipase attacks preferentially short chain fatty acids (Hofmann and Borgström, 1962) being present in Dynasan (triglyceride of myristic acid, C14).

To investigate the degradation of structurally less favorable lipids, particles made from Compritol and cetylpalmitate were used. They were identically stabilized to the Dynasan SLN (1% sodium cholate). The turbidity assay showed a very slow degradation for Compritol SLN, distinctly faster for the cetylpalmitate particles (Fig. 3) which was however not as fast as observed for the lipid Dynasan (Fig. 1). Measuring the increase in FFA confirmed this (Fig. 4). The theoretical percentage of degraded triglyceride after an incubation period of 60 min was calculated for Compritol (19.9% if only monoglycerides are formed, 9% if glycerol is the degradation product) and cetylpalmitate (28%). The molecular structure of Compritol-glyceride mixture of the long-chain fatty acid behenic acid (C22) can explain the observed low degradation. Cetylpalmitate, as a wax, can be considered as less physiologically compound than the investigated glycerides. The pancreatic lipase decomposes not only triglycerides, it is described as a hydrolase of a large number of different esters. The shorter chain length of the palmitic acid (C16) and the easy accessibility of the ester bondage in the molecule can explain the degradation velocity being faster than Compritol and slower compared with Dynasan 114. Recently, the in vitro data were confirmed by results of an in vivo toxicity study in mice. Cetylpalmitate SLN degraded very fast after repeated bolus injections and were well tolerated without increase in liver and spleen weight. Compritol degraded slowly, at a very high dose leads to a reversible fat decomposition in these organs. These results confirm the usefulness of the in vitro degradation studies for the controlled design of SLN carriers with optimized degradation and drug release performance.

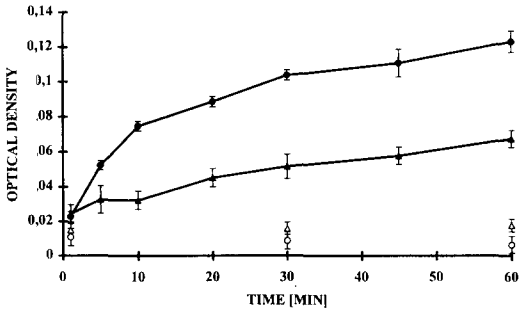

Fig. 4. The increase of free fatty acids (FFA) during the incubation of cetylpalmitate (●) and Compritol SLN (▲) (stabilizer: sodium cholate 1%) in a lipase/colipase buffer medium as a function of time. As control, the incubation of SLN in enzyme-free buffer (o, Δ) was monitored. The concentration of FFA was determined by measuring the optical density (OD) in a colour test.

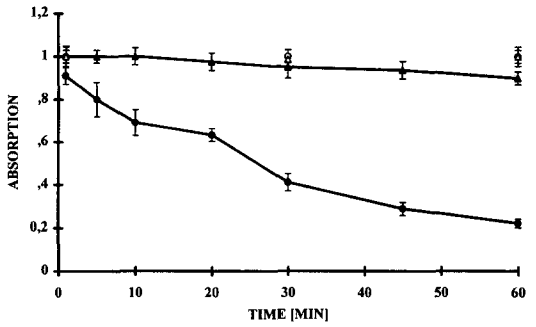

Fig. 5. Turbidity assay: degradation of Dynasan SLN stabilized with Pluronic F68 (▲) and with sodium cholate (●) as a function of incubation time in a lipase/colipase buffer medium and as control in enzyme-free buffer medium (o, Δ) (normation of absorption to 1).

To assess the contribution of the stabilizer to the degradation velocity, Dynasan SLN were stabilized with a blockcopolymer of interest for drug targeting, poloxamer 188. Particles surface-modified with this polymer showed a reduced phagocytic uptake in vitro (Rudt and Müller, 1993), in addition poloxamer is approved for intravenous use and therefore a potential stabilizer of an SLN formulation for human use (Maaßen et al., 1996). Little degradation was observed after stabilization with poloxamer in the turbidity assay (Fig. 5) and also in the corresponding FFA determination (Fig. 6). For the degradation process the lipase needs to be anchored to the particle surface. The colipase anchors the lipase close

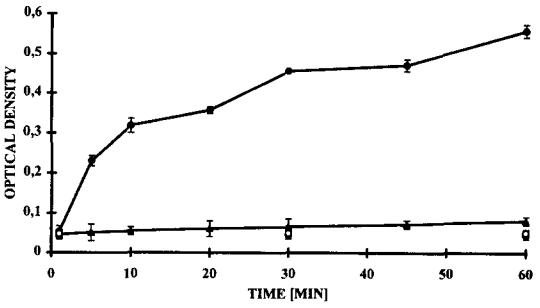

Fig. 6. The increase of free fatty acids (FFA) during the incubation of Dynasan SLN stabilized with Pluronic F68 (▲) and with sodium cholate (●) in a lipase/colipase buffer medium as a fimction of time. Control: incubation of SLN in enzyme-free buffer (o, Δ).

to, but not directly onto, the surface of the triglyceride particles. This enables lipid degradation despite the presence of bile salts (Hofmann, 1978) and explains the fast degradation of SLN stabilized with sodium cholate. Poloxamer is known as a steric stabilizer. Adsorbed on a surface, the poloxamer reduces the adsorption of proteins described as the windscreen wiper effect (Blunk, 1994). SLN particles stabilized with poloxamer 188 show a distinctly reduced adsorption of plasma proteins (Blunk et al., 1993). It is therefore assumed that the steric stabilization prevents an efficient anchoring of the lipase on the particle surface, a prerequisite for the degradation. The steric stabilization of poloxamer layers on surfaces is more effective with increasing molecular weight of the proteins. The adsorption of high molecular weight proteins is much more reduced than the ones of low molecular weight (Blunk, 1994). This supports the assumption of insufficient anchoring when considering the molecular weight of the lipase being approximately 50 000 kDa.

4. Conclusions

The turbidity assay correlated well with the chemical degradation data from FFA determination. It is therefore a fast, easy to perform and low-cost alternative for screening purposes. The lipids show distinct differences in degradation velocity. This can be exploited for modifying the release of drugs by matrix degradation. In addition, surfactants can promote degradation (e.g. bile salts in low concentrations) or almost completely inhibit degradation from basically fast degradable lipids (poloxamer, Dynasan SLN). The nature of the lipid and of the stabilizer are efficient tools to control SLN degradation over a broad range. It opens even the perspective, to prepare slowly or little biodegradable SLN for GIT drug delivery from GIT-degradable lipids by choosing the appropriate stabilizer (e.g. poloxamer). In addition, the lipid can solely be chosen with regard to optimum drug incorporation properties when controlling the degradation via the surfactant.

References

Amselem, S., Domb, A.J. and Avling, C.R., Lipospheres as a vaccine carrier system: effects of size, charge, and phospholipid composition. *Vaccine Res.*, 1(4) (1992) 383–395.

Blunk, T., Plasmaproteinadsorption auf kolloidalen Arzneistoffträgern. *PhD Thesis*, University of Kiel, 1994.

Blunk, T., Hochstrasser, D.F., Sanchez, J.-C., Müller, B.W. and Müller, R.H., Colloidal for intravenous drug targeting: plasma protein adsorption patters on surface-modified latex particles evaluated by 2-D polyacrylamide gel electrophoresis. *Electrophoresis*, 14 (1993) 1382–1387.

Boltri, L., Canal, T., Esposito, P. and Carli, F., VectorPharma International S.p.A., Trieste Italy, Relevant factors affecting the formulation and growth of lipid nanospheres suspensions. *Eur. J. Pharm. Biopharm.*, 41(1) (1995) 70–75.

Borgström, B., On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. *J. Lipid Res.*, 16 (1975) 411–417.

Gasco, M.R. and Morel, S., Lipospheres from microemulsions. *II Farmaco*, 45 (1990) 1127–1128.

Hofmann, A.F., Lipase, colipase; ainphipathic dietry proteins, and bile acids: new interactions at an old interface. *Gastroenterlogy*, 75 (1978) 530–532.

Hofmann, A.F. and Borgström, B., Physico-chemical state of lipids in intestinal content during their digestion and absorption. *Fed. Proc.*, 21 (1962) 43–50.

Keller, M.P., Buri, P. and Charollais, E., Importance des lipases du tractus digestif pour l'hydrolyse des exipients lipidiques et l'enzymothérapie de substitution. *Pharm. Acta Helv.*, 67 (2) (1992).

Lairon, D., Nalbone, G., Lafont, H., Leonardi, J., Domingo, N., Hauton, J.C. and Verger, R., Possible roles of bile lipids and colipase in lipase adsorption. *Biochemistry*, 17(24) (1978) 5263–5269.

Lairon, D., Nalbone, G., Lafont, H., Leonardi, J., Vigne, J.L., Chabret, C., Hauton, J.C. and Verger, R., Effects of bile lipids on the adsorption and activity of pancreatic lipase on triglycerol emulsions. *Biochim. Biophys. Acta*, 618 (1980) 119–128.

Maaßen, S., Schwarz, C., Mehnert, W. and Müller, R.H., Solid lipid nanoparticles (SLN) formulation for human use: interaction with human granulocytes. *J. Control. Release*, (1996) submitted.

Momsen, W.E. and Brockman, H.L., Effects of colipase and taurodeoxycholate on the catalytic and physical properties of lipase B at an oil-water interface. *J. Biol. Chem.*, 251 (1976) 378.

Müller, R.H., Lherm, C., Herbort, J. and Couvreur, P., In vitro model for the degradation of alkylcyanoacrylate nanoparticles. *Biomaterials*, 11 (1990) 590–595.

Müller, R.H., Lherm, C., Herbort, J., Blunk, T. and Couvreur, P., Alkylcyanoacrylate drug carrier: I. Physicochemical characterisation of nanoparticles with different alkyl chain length. *Int. J. Pharm.*, 84 (1992) 1–11.

Müller, R.H. and Lucks, J.S., *German Patent Application P 4131562.6*, 1991.

Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Mühlen, A., Weyhers, H., Freitas, C. and Rühl, D., Solid lipid nanoparticles (SLN)-an alternative colloidal carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm.*, 41(1) (1995) 62–69.

Rudt, S. and Müller, R.H., In vitro phagocytosis assay of nano and microparticles by chemiluminescence: II. Effect of surface modification by coating of particles with poloxamer on the phagocytic uptake. *J. Control. Release*, 25 (1993) 51–59.

Schwarz, C., Mehnert, W., Lucks, J.S. and Müller, R.H., Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterisation and sterilisation. *J. Control. Release*, 30 (1993) 83–96.

Siekmann, B. and Westesen, K., Submikron-sized parenteral carrier systems based on solid lipids. *Pharm. Pharmacol. Lett.*, 1 (1992) 123–126.

Wako Chemicals, instruction for NEFA C test, 1993.

Wallis, K.H. and Müller, R.H., Comparative measurements of nanoparticle degradation velocity using an accelerated hydrolysis test. *Pharm. Ind.*, 55 (1993) 168–170.

Weyhers, H., Preparation and characterisation of solid lipid nanoparticles for drug targeting. *PhD Thesis*, Free University of Berlin, 1995.

Weyhers, H., Lück M., Mehnert W. and Müller, R.H., Solid Lipid Nanoparticles (SLN) for controlled delivery of hydrophilic drugs. *1st World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Budapest, 1995.

Wollesen, C., Untersuchungen zur Wirkung des Appetitzüglers Fenfluramin auf die Lipidresorption bei der Ratte. *PhD Thesis*, University of Kiel, 1981.

zur Mühlen, A., Feste Lipid Nanopartikel mit prolongierter Wirkstoffliberation-Herstellung, Langzeitstabilität, Charakterisierung, Freisetzungsverhalten und-Mechanismen. *PhD Thesis*, Free University of Berlin, 1996.