Atomic Force Microscopy Studies of Solid Lipid Nanoparticles

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Purpose. Solid Lipid Nanoparticles (SLN) are an alternative carrier system for the controlled delivery of drugs. In most cases prednisolone loaded SLN show a biphasic release behaviour. The initial phase is characterised by a fast drug release, which is followed by a sustained drug release over several weeks.

Methods. The particles are produced by high pressure homogenisation of a lipid (e.g. compritol, cholesterol) dispersed in an aqueous surfactant solution. In this study atomic force microscopy was used to image the original unaltered shape and surface properties of the particles. The crystallinity of the nanoparticles was investigated by differential scanning calorimetry.

Results. The AFM investigations revealed the disc like shape of the particles. From differential scanning calorimetry data it can be concluded that the particle core is in the crystalline state. Additionally it was proven that the particles are surrounded by a soft layer.

Conclusions. Thus it is conceivable that the fast initial drug release during in vitro dissolution tests takes place by drug release of the outer non-crystalline layers of the particles. The following sustained drug release can be assigned to the predisolone release of the inner crystalline particle layers.

KEY WORDS: atomic force microscopy; solid lipid nanoparticles; controlled drug delivery; prednisolone.

INTRODUCTION

SLN are produced as drug carrier systems for topical, oral and parenteral administration of drugs (1). The system consists of Solid Lipid Nanoparticles in the nanometer range, which are dispersed in water or in an aqueous surfactant solution. The particles are made of physiological and biodegradable lipids, which leads to the low systemic toxicity of these carrier systems. The use of high pressure homogenisation as production method yields dispersions with low contents of particles in the micrometer range (2). Therefore the SLN fulfill the special requirements for intravenous administration. The hydrophilic surface of SLN created by poloxamer makes it a potential candidate for a reduced uptake of macrophages of the reticuloendothelial system. The production method provides also the possibility of large industrial scale production. The release of most drugs from fat emulsions is very fast due to the distribution of the drug between the oil droplets and the large volume of the blood. In contrast, SLN consist of a solid matrix which reduces the drug mobility and prevents drug leakage from the system. Similar to polymeric nanoparticles (3) the SLN carrier system allows the controlled release of drugs. As reported with prednisolone a sustained release of SLN over several weeks was achieved (4). Atomic force microscopy (AFM) (5) was applied as a new tool to image the surface of nanoparticles. This technique utilises the force acting between a surface and a probing tip resulting in a spatial resolution of up to 0.01 nm for imaging. A striking advantage of AFM is the simple sample preparation as no vacuum is needed during operation and the sample does not need to be conductive. Therefore originally hydrated, solvent containing samples can be analysed. Thus AFM gained some recent attention in the field of biological and pharmaceutical sciences. For example imaging of fibrinogen polymerisation (6), the budding of a virus of an infected cell (7), the in vitro degradation of polymer surfaces (8) and polymer nanoparticles (9) were performed. It has been reported for biological compounds that it is a sufficient sample preparation to place a drop of a solution or dispersion of a sample on a washed microscope slide or on a mica substrate (6, 10). The atomic force microscope obtains images fast enough (about twenty seconds per image) to allow the observation of in situ processes occurring at interfaces.

MATERIALS AND METHODS

Compritol 888 ATO was provided by Gattefossé (Weil am Rhein, Germany), poloxamer 188 by ICI (Wilton, UK), prednisolone by Ferring (Kiel, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

Solid Lipid Nanoparticles were prepared as described previously by cold or hot dispersion technique (11). The SLN formulations used in this investigations consisted of 5% lipid, 1–2.5% poloxamer 188, 0.05% prednisolone and distilled water ad 100%.

During imaging the force microscope was either operating in the contact or non-contact mode. These modes differ mainly by the sensitivity of the tip to the respective forces used for imaging (5). During contact mode imaging the tip touches the sample surface which may result in alterations of the sample surface, such as the removal of particles from the area under investigation. However these problems can be avoided by non-contact imaging, as the forces acting between tip and sample are smaller by a few orders of magnitude (5). In this study a commercial AFM was used (Topometrix TMX 2000, Sta. Clara, USA). The measurements were performed under ambient air conditions. Contact imaging has been done with microfabricated Si_3N_4 tips attached to cantilevers (spring constant of 0.032 Nm^{-}); the tip is a four sided pyramid with a height of 3 μm. For non-contact imaging triangular Si tips were chosen with a height of 8–12 μm. Typical resonance frequencies of these tip cantilever systems were found to be about 200 kHz.

In addition to topography imaging further information about the mechanical properties of an investigated system can be gained if the force acting between tip and sample is monitored while the tip is approaching the sample surface (force distance curves). Prior to being in contact tip and sample do not interact. The tip is pulled rapidly onto the surface as soon as the contamination layers (naturally adsorbed water) of tip and sample overlap. Further attractive forces support this process until the tip is experiencing repulsive forces. The total bending of the tip towards the surface is a measure for the total thickness of the adsorbed layers. Once in the repulsive regime the tip is moving upward as the sample is raised further. The response of the cantilever reveals the hardness of the sample. On an ideally

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rigid sample the tip movement parallels the sample movement completely. In contrast, a soft sample experiences deformation while it is pushed into the tip resulting in a smaller displacement of the tip relative to the sample.

For AFM investigations it is sufficient to fix the particles on an appropriate smooth substrate surface to allow an unambiguous distinction between the particles and the surface. First the SLN dispersion was diluted in distilled water (1 μl ml^{-1}). A 5 μl drop of this dilution was deposited on a silicon wafer surface. The water phase of the SLN dispersion was removed by heating the silicon substrate to 30°C. Due to this gentle treatment it can be assumed that no or little alterations of the specimen occur which results in a much easier interpretation of images (12).

For non-contact AFM operation it was sufficient to place the nanoparticles on a standard polished silicon wafer surface with a roughness below 1 nm. In case of contact mode operation it was necessary to develop a new way of fixing the nanoparticles as the deposition of the particles on such a smooth surface resulted in their removal by the tip. Thus prior to drop evaporation the silicon wafer surface was roughened to values of up to 10 nm by etching the wafers in hydrofluoric acid and ammonium fluoride.

Particle size analysis was performed by photon correlation spectroscopy (Malvern Zetasizer IV, Malvern, UK). The crystalline status was analysed by a differential scanning calorimeter (TA 2300, Mettler Toledo GmbH, Gießen, Germany) The samples were heated from 40°C to 90°C and cooled down again to 40° at a scan rate of 5°C min^{-1}. 15 mg of the SLN dispersion or 3 mg of the bulk material were weighed in aluminium pans. As reference an empty pan was used.

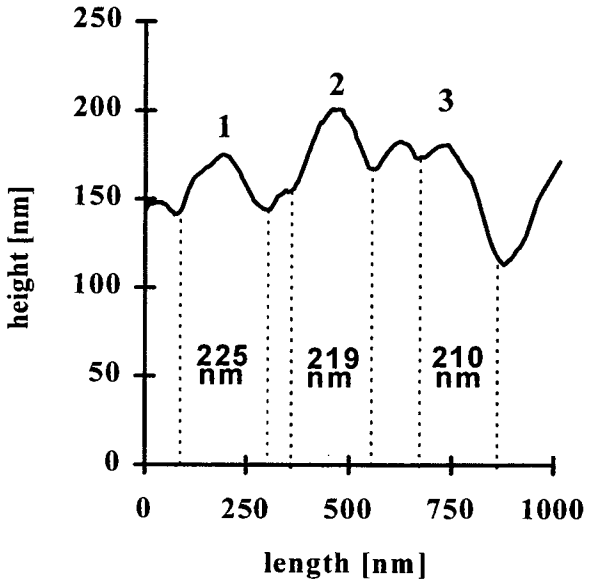
RESULTS AND DISCUSSION

Comparison Between the Results of PCS Measurements and AFM Results

In AFM images protruding particles with sudden height changes at their edges may appear broadened due to the finite dimension of the tip (10). At such a sudden height change different regions such as the sides of the tip rather than the tip end may interact with the sample. For SLN these interactions can be observed at the side parts of the particles resulting in a fuzzy image at the edges of the particles (for an example see fig. 1). To minimise this inaccuracy for the quantitative analysis of the particles the 'Full Width at Half Maximum' values of the particle were used to determine the sizes of the particles. For adjacent particles as shown in figure 1 the sizes are indicated by the gaps between the particles. In figure 1 analysed particles possess diameters ranging from 210 nm up to 225 nm. Photon correlation spectroscopy (PCS) investigations of this SLN dispersion reveal a mean particle size of 160 nm and a polydispersity index of 0.268. Thus the results of particle size measurements by PCS and AFM are in the same magnitude of size.

Characterisation of SLN

Cholesterol and compritol were employed as matrix materials. Because of the high melting point of cholesterol (148°C) the particles were produced by the cold homogenisation technique. During the production process the lipid matrix remained mainly in the solid state despite possible high but short temperature peaks occurring in the high pressure homogeniser. The



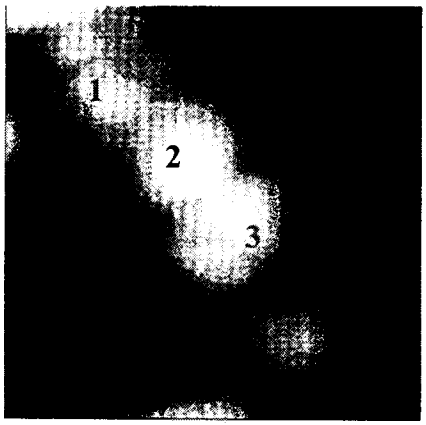


Fig. 1. Image of compritol nanoparticles produced by homogenisation of a pre-emulsion by a temperature (90°C) above the melting point of the lipid. The formulation consists of 5% compritol, 2.5% poloxamer 188 and 92.5% water. The size analysis (top of figure) shows the width and the height of the imaged particles (marked with numbers) (bottom of figure). Imaging was performed by using the non-contact mode.

cold homogenisation is less effective in dispersing the lipids. Simultaneously applying a higher number of homogenisation cycles yielded particles with a mean diameter ranging from 800 nm to 1,000 nm (PCS measurements). The imaged particles possess a disc like shape. On the particle surface detailed structures are visible (Fig. 2).

Compritol has a melting point of about 72.3°C and was therefore chosen because the dispersions can be produced by the hot and alternatively by the cold homogenisation technique. Compritol consists of about 28–32% tribehenate, 52–54% dibehenate and 12–18% monobehenate. By varying the applied production methods and temperatures different particle sizes were obtained (2,11).

In contrast to cholesterol particles the imaged particles show no detailed structure on the particle surface (Fig. 3, A), appearing rather like amorphous PLGA and PEG-PLGA poly-

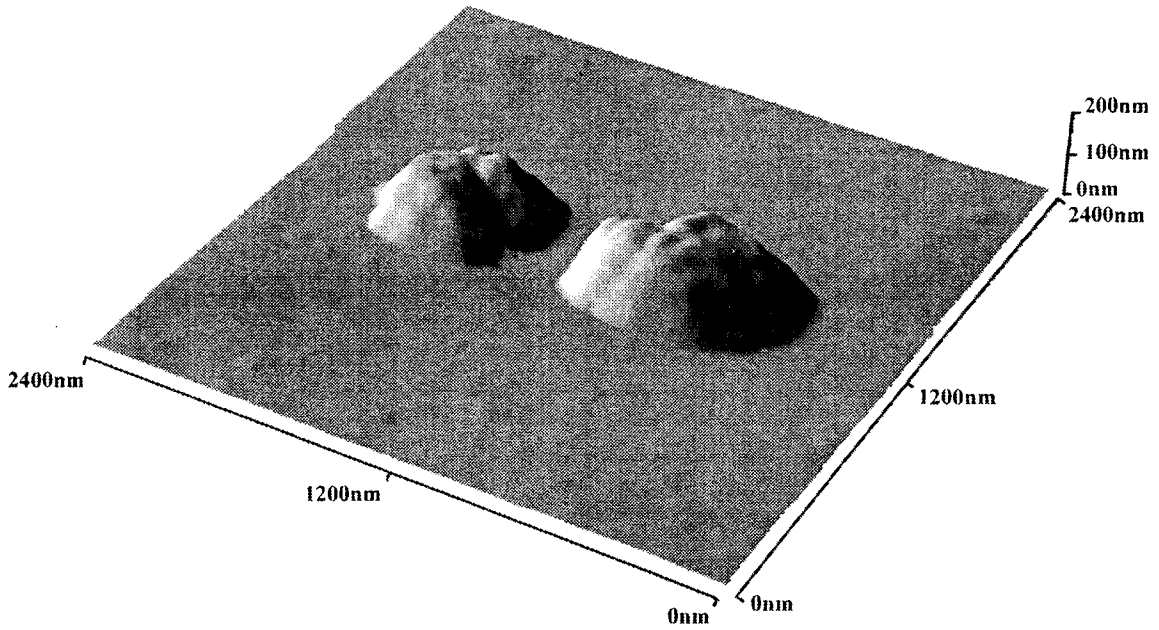


Fig. 2. This image shows prednisolone loaded cholesterol based particles, which were stabilised with 2.5% poloxamer 188. Structures at the edges are most likely to tip artefacts and should be ignored. The particles were analysed 3 days after production by using the contact mode.

mer nanoparticles (9). Of course an unambiguous distinction between crystalline and amorphous particles by AFM only is hardly possible. To investigate the crystalline structure of the nanoparticles DSC thermograms from compritol SLN and pure compritol were monitored (Fig. 4). Pure compritol shows one melting peak with a maximum at 70.1°C and a melting enthalpy of about 133 J/g, which are comparable with the melting point and enthalpy of the β′ modification of pure tribehenate (74.8°C, 152 J/g) (13). The observed depression of melting enthalpy and melting point are caused by the high content of diglycerides, which leads to a high number of lattice imperfections (14). Normally triglycerides possess a crystal structure due to the β modification. However diglycerides can avoid the transformation to this modification. It is reported that mixtures of tri- and diglycerides occur therefore in the β′ (13) or βi modification (15). The βi modification is characterised as an intermediate form between the β′ modification and β modification (16). It can be assumed that the melting of pure compritol can be attributed to the melting of the β′ or βi modification.

Upon heating of the compritol SLN dispersion a single broad endothermic peak was obtained (Fig. 4, C). The melting point of the SLN is about 3°C lower than the melting point of the bulk material. Reasons for the melting point depression can be the small size of these carriers (17) or the high content of lattice imperfections (18). In comparison to the DSC thermogram of pure compritol, the melting point of compritol SLN can be assigned to the β′ or βi modification.

The melting enthalpies of SLN (129 J/g) and bulk material (133 J/g) are almost identical, therefore most of the particles must be recrystallised after the production process. Focusing on the cooling curve two exothermic peaks occurred (Fig. 4, D) which do not appear when analysing the bulk material (Fig. 4, B). These results and especially the cooling curve, which is normally a more sensitive method to detect polymorphic forms

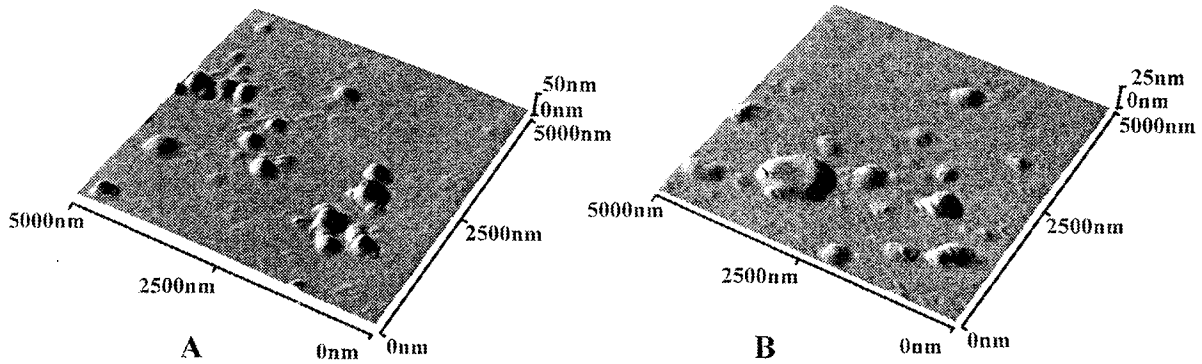


Fig. 3. AFM image of drug loaded SLN. The dispersion consists of 0.05% prednisolone, 5% compritol, 1% poloxamer, 92.45% water. Imaging was performed by using the non-contact mode 2 days after production (A) and after exposure of the SLN to an aqueous medium for 1 week (B). In contrast these particles appear more flat.

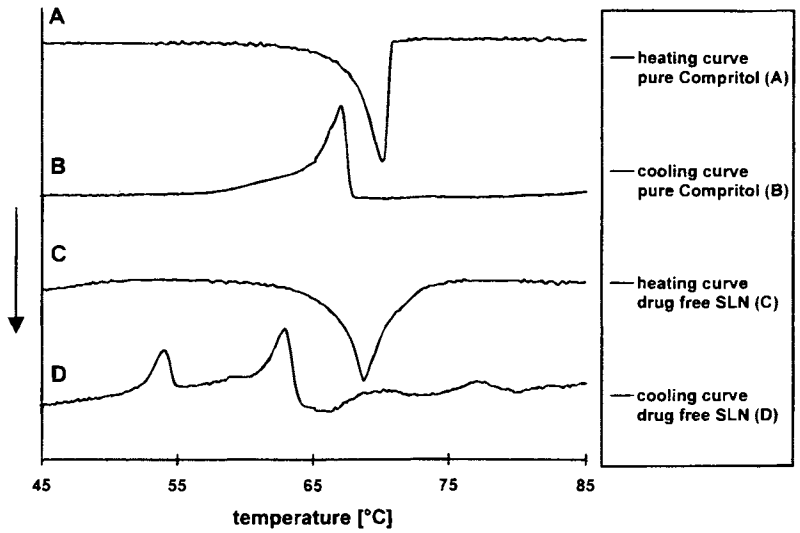


Fig. 4. DSC thermograms of pure compritol (curve A, B) and compritol based SLN (three days after the production) stabilised with 0.5% poloxamer 188 (curve C, D). Heat of fusion of pure compritol (curve A) 133 J g^{-1} and of the compritol SLN matrix 129 J g^{-1}.

(16), indicate that compritol SLN recrystallised in two different polymorphic forms. Reheating the solidified sample led to the same curve as the first heating scan (data not shown). However it can be assumed that the broadening of the melting peak was induced by the different polymorphic forms. Like it is the case of compritol based sprayed lipid micropellets (19) the compritol SLN matrix is composed of different polymorphic forms, but mainly the matrix consists of the β′ or βi modification.

The internal structure of the tripalmitate SLN were investigated by Transmission Electron Microscopy (TEM) of freeze fractured particles (20). The imaged lipid crystals possess a plate like structure (20). In contrast to the TEM investigations of freeze fractured SLN, AFM visualises the topography of the unaltered particles. The AFM scans of compritol SLN reveal a disc shape of the particles (e.g., in fig. 3), which could be induced by the plate like structure of the lipid crystals.

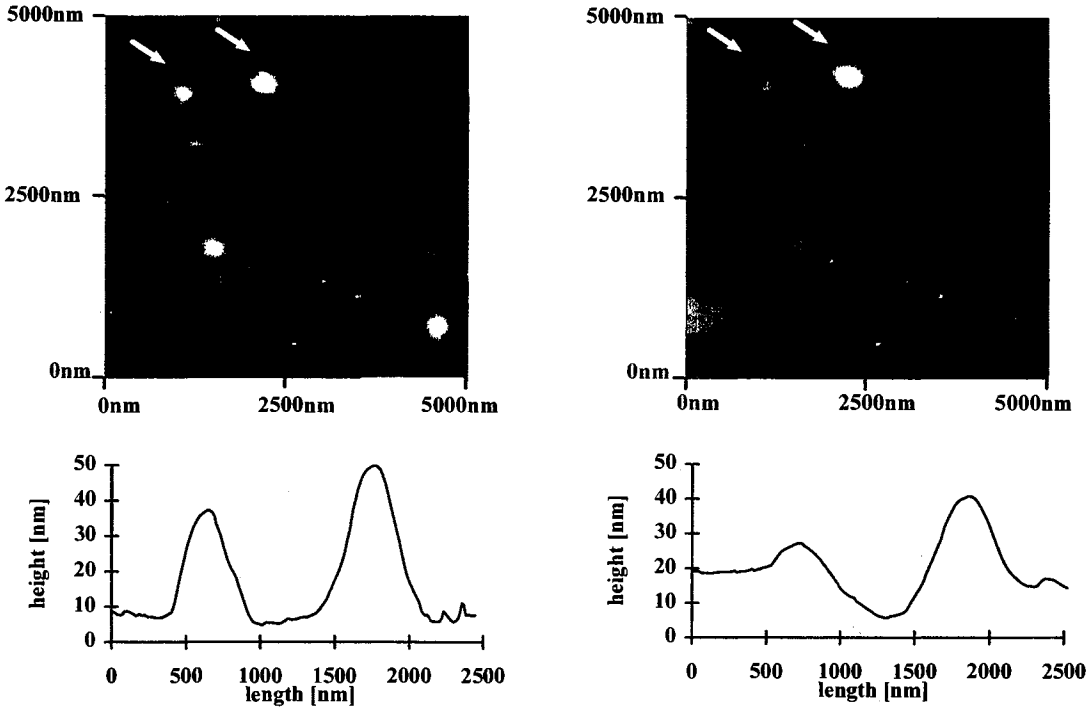


Fig. 5. Image of compritol nanoparticles produced by hot homogenisation. Imaging was performed by using the contact mode. On the left side are the image and the size analysis of the particles (marked by white arrows) after the first scan. On the right side are the image and the size analysis of the same particles (marked by white arrows) after the three successive scans. The formulation is composed of 5% compritol, 2.5% poloxamer 188 and 92.5% water.

By using the contact mode compritol SLN experience deformations of the scanned particles during successive measurements (Fig. 5). A size analysis of two selected particles as they were imaged in a first scan show that the chosen particles are about 37 nm and 50 nm high and about 350 nm and 400 nm wide (Fig. 5, left side). Three successive scans later these particles possess a height of about 27 nm and about 41 nm and a width of 420 nm and 500 nm (Fig. 5, right side). The fact that the weak forces of the tip during successive measurements are sufficient to deform the particle indicates that the outer layers of the particles possess a soft character. The AFM technique bears the possibility to quantify the hardness of the scanned surface. Therefore force distance curves of the particle surface were taken and compared with the results monitored on the uncovered silicon surface.

Representative force distance curves obtained during the approach of the sample towards the tip are displayed in figure 6. The top of figure 6 shows the tip response to the approaching Silicon wafer surface. Before jumping to contact the tip is free and raising the sample has no influence (Fig. 6, I). As soon as the contamination layers of tip and surface interact, the tip is pulled towards the surface (Fig. 6, II). Once in contact the tip follows the "hard" silicon surface in its upward movement immediately (Fig. 6, III).

For comparison a typical response curve obtained on an SLN is shown at the bottom of figure 6. A striking difference to the curve obtained on silicon can be found in the much larger

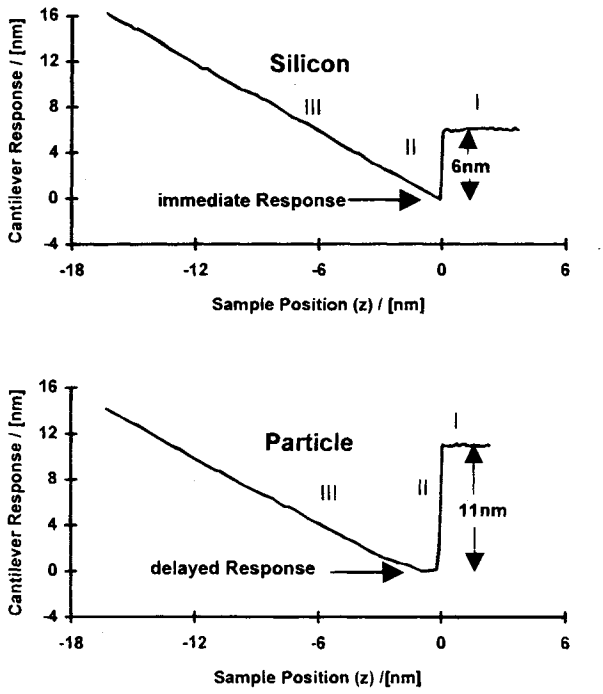


Fig. 6. Shown is the tip response during approach of the sample. The bottom curve has been obtained on a particle, the top curve on Silicon. First the tip is free and not influenced by the approaching surface (I). Once the contamination layers of tip and sample overlap the tip jumps to contact (II). Then the tip rises again with the upward movement of the sample (III). The upward motion is influenced by the hardness. For Compritol SLN (stabilised by 2.5% Poloxamer 188) a 1 nm deformation of the particle leads to a horizontal slope of the curve after jumping to contact.

change of deflection while the tip is pulled to contact. That indicates that a thicker contamination layer covers the hydrophilic nanoparticles compared to the hydrophobic silicon. From the total deflection values, which can be calibrated by the contact slope of the silicon response curve, the thickness of the contamination layer can be calculated to be about 11 nm. Being in contact the sample has to be raised one further nm before the deflection increases again. This indicates a deformation of the sample once the particle is pushed into the tip, revealing the soft character of the shell of the particle.

It can be expected that the outer layers are composed of the more hydrophilic mono- and diglycerides. These effects lead to a higher hydrophilicity in the outer layers of the particles, which is additionally intensified by the surfactant. In these layers water can easily be incorporated affecting the soft character of the matrix.

The alterations during the measurement can be avoided by using the non-contact mode (Fig. 3, A). Therefore for imaging the particles the non-contact mode is more appropriate. Additionally the removal of the particles during measurement can be avoided and therefore the sample preparation is much easier.

Characterisation of Drug Loaded Particles

Prednisolone loaded compritol SLN, which were produced by the hot homogenisation technique, show a fast initial drug release of 13–78% of the incorporated drug in the first few minutes of the in vitro dissolution test, followed by a prolonged drug release over several weeks (4). The mechanism leading to the fast initial drug release is caused by the enrichment of drug in the outer shell of the particle (4). As characterised above the outer layers possess a soft and relative hydrophilic character, in which the drug is easily incorporated to a high amount and can be easily released as well. Therefore the enriched drug in the outer layer causes the burst release after dilution in the dissolution testing system.

This fast initial drug release is followed by a slow sustained prednisolone release over a monitored period of 5 weeks (4). Imaged particles, which were exposed 2 weeks to the dissolution medium (Fig. 3, B), possess the same disc-like shape as the original SLN (Fig. 3, A). However they appear more flat.

CONCLUSIONS

This study demonstrates the ability of the AFM—especially if operating in the non-contact mode—to image the morphological structure of solid lipid nanoparticles. The size of the visualised particles are of the same magnitude compared with the results of PCS measurements. The AFM investigations revealed the disc like structure of the particles. DSC thermograms confirmed that most of the particles are crystallised in the β′ or βi modification and that they possess a high degree of crystallinity. AFM images indicate that the crystalline particles are surrounded by soft layers. The softness of these layers was proved by form alterations, which occurred if they were imaged successively by contact AFM. By monitoring force distance curves the hardness of the particle surface compared to the silicon surface was quantified. In contrast to the silicon surface a form deformation of about 1 nm occurred when the particle was pushed into the tip.

It is conceivable that prednisolone can be easily incorporated in these layers, but also easily released which leads to the fast initial drug release during the in vitro dissolution test.

After exposing compritol drug loaded particles to an aqueous dissolution medium the particles maintain the disk like shape although they loose in thickness.

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