# 3 Materials and Methods

## *3.1 Materials*

### 3.1.1 Cyclosporine

#### 3.1.1.1 Therapeutic indication

Cyclosporine is the most widely used immunosuppressant drug to prevent transplant rejection. It has been studied in transplants of heart, kidney, lung, pancreas, liver and skin transplants (Sneader 2005). Apart from in transplant medicine, cyclosporine is also used in cases of psoriasis and infrequently in rheumatoid arthritis and related diseases. Cyclosporine has also been investigated for the use in many other autoimmune disorders. Lately the treatment of ulcerative colitis was reported to be beneficial (Krauze et al. 2002).

#### 3.1.1.2 History and commercialisation

The immuno-suppressive effect of cyclosporine was discovered on January 31, 1972, by employees of Sandoz (now Novartis). The newly discovered drug was subsequently approved and was first launched in 1983 under the brand name Sandimmun. This formulation being a solution of dissolved cyclosporine in corn oil yielded only a poor bioavailability with tremendous intra individually variations. Cyclosporine was re-formulated as a microemulsion pre-concentrate, yielding higher bioavailability and less intra individually variation. The improved formulation was launched 11 years later under the trade name Sandimmun Optoral/Neoral. Nowadays also generic products are marketed by other companies, e.g. Hermal, Abbott Laboratories, Gengraf and Sangstat. Cyclosporine for topical application is marketed since 2002 under the name Restasis. It is an emulsion for the treatment of keratoconjunctivitis sicca. Annuals sales are around 1 billion US$ (Novartis Pharma GmbH 2004; Sneader 2005).

#### 3.1.1.3 Mode of action and metabolism

Cyclosporine binds to the cytosolic protein cyclophilin (also immunophilin) of immunocompetent lymphocytes, especially T-lymphocytes. This complex of cyclosporin and cyclophylin inhibits calcineurin, which under normal circumstances is responsible for activating the transcription of interleukin-2. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector T-cells.

The oral absorption is thought to be passive. There is only a small absorption window of cyclosporine in the upper small intestine. Cyclosporine is a substrate for p-glycoprotein and cytochrome P450 3A4, both present in the intestine. This fact is the main subject to the considerable interindividual variation in bioavailability. Cyclosporine is metabolised in the liver by the cytochrome P450 3A4 system. The distribution depends not only on physicochemical characteristics, but also on biological carriers such as lipoproteins, erythrocytes in blood and its binding protein cyclophilin. Its half live is about 24 hours. The elimination is mainly bilary, other routes have been described, but have not much impact. Pharmacokinetic parameters of cyclosporin are highly variable and depend on factors such as age, the physical condition of the patient, type of organ transplant or comedication. The therapeutic range and dosage of cyclosporin are therefore highly dependent on many individual parameters in patients (Hassan 1987; Fahr 1993; Mutschler et al. 2001).

#### 3.1.1.4 Adverse effects and interaction

The treatment of cyclosporine is mostly associated with a number of serious adverse drug reactions (ADRs) and adverse drug interactions. ADRs can include gum hyperplasia, convulsions, peptic ulcers, pancreatitis, fever, vomiting, diarrhea, confusion, breathing difficulties, numbness and tingling, pruritus, high blood pressure, potassium retention and possibly hypercalemia, kidney and liver dysfunction (nephrotoxicity & hepatotoxicity), and obviously an increased vulnerability to opportunistic fungal and viral infections. Reasons for ADRs are many fold and up to now not fully understood. However kidney and liver dysfunctions are caused by the induction of oxidative stress due to cyclosporine itsself (Wolf et al. 1997; Rezzani 2004) Renal side effects of cyclosporine are dose-related, where dosages of less than 5 mg/kg/day, rarely cause renal side effects (Fahr 1993). Cyclosporine interacts with a wide variety of other drugs and other substances. Mostly caused due to cytochrome P450 3A4 interactions (Mutschler et al. 2001).

#### 3.1.1.5 Properties

Cyclosporine is a cyclic non ribisomal peptide of 11 amino acids (undecapeptide). It is produced by the fungi Hypogladium inflatum gams and Tolypocladium inflatum (Sneader 2005).

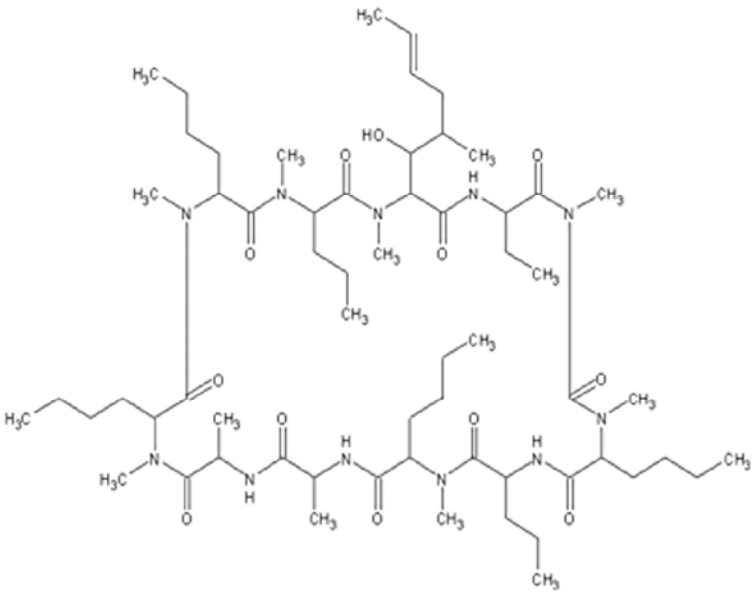

**Figure 3-1: Cylosporine structure after (Rote Liste 2003)**

## Cyclosporine monograph

| | |
|---|---|
| Synonyma | Ciclosporin (INN), *[R-[R*,R*-(E)]]-cyclic(L-alanyl-D-alanyl*-N-*methyl-L-leucyl*-N-*methyl-L-leucyl*-N-*methyl-L-valyl-3-hydroxy*-N,*4-dimethyl-L-2-amino-6-octenoyl-L-α-amino-butyryl*-N-*methylglycyl*-N-*methyl-L-leucyl-L-valyl*-N-*methyl-L-leucyl)* |
| Indication | immunosuppressant |
| Formula | $C_{62}H_{111}N_{11}O_{12}$ |
| CAS.-No. | 59865-13-3 |
| Molecular weight | 1202.61 |
| Properties | White crystaline powder |
| Solubility data | Less than 0.4µg/ml in water, soluble in ethanol, isopropylic alcohol |
| Melting point | 148 -151 C |

Extraordinary properties of cyclosporine are its inversingly solubility to the temperature (Augustijns et al. 2000) and its reported liquid crystalline properties (Lechuga-Ballesteros et al. 2003). Cyclosporine was purchased from Chemos GmbH (Regensstauff, Germany).

### 3.1.2 Stabilisers with inhibition of p-glycoprotein

#### 3.1.2.1 TPGS

D-α-Tocopheryl-Polyethylenglykol-1000-succinat (TPGS) is a watersolube vitamin E derivate, which was originally developed as food additive. TPGS has amphiphilc properties and is therefore used as emulsifier for nutraceuticals and pharmaceuticals (Lehr 2005). Later it was used for the treatment of patients with vitamin E deficit and disabled fat absorption (Sokol et al. 1987). Its ability to inhibit p-glycoprotein and to increase the bioavailability of cyclosporine was first reported in 1996 (Chang et al. 1996; Dintaman and Silverman 1999). The best inhibitory effect was found for D-α-Tocopheryl-Polyethylenglykol-1000-succinat, other TPGS-types with lower or longer chains of polyethylene glycol were studied to be less effective for the inhibition of p-glycoprotein (Lehr 2005).

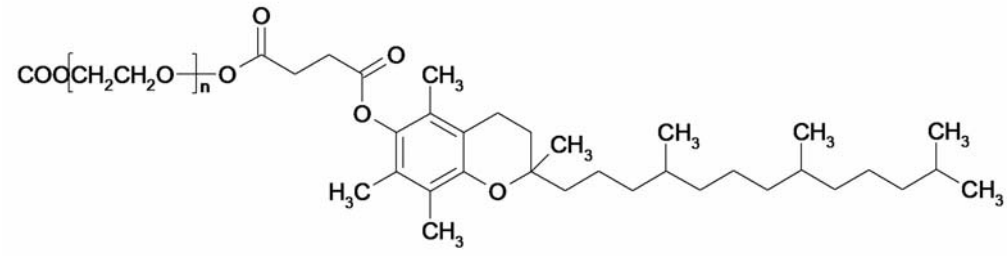

**Figure 3-2: Chemically structure of TPGS after (Eastman 2002)**

**TPGS monograph**

| | |
|---|---|
| Synonyma | vitamin E polyethylene glycol succinate (USP)D-α-Tocopheryl-Polyethylenglykol-1000-succinat (INN) |
| Indication | Surfactant, p-glycoprotein inhibitor |
| Formula | $C_{34}H_{70}O_7(C_2H_4O)_{1000}$ |
| CAS.-No. | 9002-96-4 |
| Molecular weight | 45416 |
| Properties | light-yellow solid (waxy) |
| Solubility data | soluble in water |
| Melting point | 36°C |
| HLB | 13 |

TPGS was kindly provided from Eastman (Kingsport, USA)

#### 3.1.2.2 Other surfactans with p-glycoprotein inhibition

Sodium dodecyl sulphate (SDS); polysorbate 80 (Tween 80) (Sigma Aldrich Chemie GmbH, Steinheim, Germany); polyoxyethylene block copolymers: Poloxamer 188 (Lutrol F 68) and Poloxamer 407 (Lutrol F 127) (BASF AG, Ludiwigshafen, Germany); polyoxyethylene glycerol trioleate (Tagat TO) (Degussa, Frankfurt, Germany); glycerol-polyethylene-ricinoleat pH-range 6.0-8.0 (polyoxyl-35-castor oil, Cremophor EL) (Sigma Aldrich Chemie GmbH, Steinheim, Germany)

### 3.1.3 Surfactants without inhibition of p-glycoprotein

Sodium glycocholate (Sigma Aldrich Chemie GmbH, Steinheim, Germany); Dehyquart A-CA (Cognis, Düsseldorf, Germany); chitosan (degree of deacytylation 86.5%, viscosity 465, MW 250k) from (Primex Ingredients ASA, Bremen, Germany); chitosan chloride (Protasan CL 213) (Novamatrix, Drammen, Norway); polyvinyl pyrolidone (PVP MW 25k) polyvinyl pyrolidone (PVP Mw 40k) (Sigma Aldrich Chemie GmbH; Steinheim, Germany); soya lecithin (Phospholipon S75) and egg lecithin (Phospolipon E80) (Nattermann, Phospholipid GmbH, Köln, Germany); saponin from quillaja bark (Sigma Aldrich Chemie GmbH, Steinheim, Germany); polysorbate 20 (Tween 20) (Sigma Aldrich Chemie GmbH, Steinheim, Germany); emulsifying cetylsteary alcohol type A (Lanette N) and cetylstearyl alcohol (Lanette O) (Caesar & Loretz GmbH, Hilden, Germany)

### 3.1.4 Quasie-emulsifiers for viscosity enhancement

Xanthan (Kelco, Atlanta, USA); methylcellulose Typ 400, hydroxyethylcellulose (Tylose® H300), hydroxypropylcellulose (KLUCEL GF®) (nominal viscosity 300 mPas) (Caesar & Loretz GmbH, Hilden, Germany), highly disperse silicium dioxide (Aerosil 200) and polyacrylic acid (Carbopol 940) (Degussa AG, Hanau-Wolfgang, Germany); gelatine A, gelatine B (Naumann-Gelatine und Leim GmbH, Memmingen, Germany); Aeroperl 300/30 pharma grade (Degussa AG, Hanau-Wolfgang, Germany)

### 3.1.5 Peppermint oil

Peppermint oil is obtained through steam distillation of the fresh above-ground parts of peppermint (Mentha x piperita), which is a cross (hybrid) between spearmint and water mint. Essential oil constituents are cineol, isomenthone, liminene, menthofuran, menthol, menthone, menthyl acetate, terpenoids. Peppermint oil has been used historically for numerous health conditions, including common cold symptoms, cramps, headache, indigestion, joint pain, and nausea. Today it is mainly used for the treatment of irritable bowel syndrome and for dyspepsia (Hiki et al. 2003; Grigoleit and Grigoleit 2005; Hadley and Gaarder 2005). Other indications are anorexia, antiviral, arthritis, asthma, bile duct disorders, bronchial spasm, cancer, chicken pox, cholelithiasis (gallstones), colonic spasm (during colonoscopy or barium enema), common cold, cough, cramps, dysmenorrhea (menstrual pain), enteritis, fever, fibromyositis, gallbladder disorders, gas (flatulence), gastrointestinal disorders, gastritis, head lice (Pediculus humanus capitus), ileus (post-operative), inflammation of oral mucosa, influenza, intestinal colic, laryngeal spasm, local anesthetic, morning sickness, motility disorders, musculoskeletal pain, myalgia (muscle pain), neuralgia (nerve pain), postherpetic neuralgia, pruritus (itching), rheumatic pain, sun block, tendonitis, toothache, tuberculosis, urticaria (hives).

Though to the activity is manifold, but the mode of action is not fully understood. Intense research on this compound is ongoing. Absorption studies showed that peppermint oil is relatively rapidly absorbed after oral administration and that it is eliminated mainly via the bile (Grigoleit and Grigoleit 2005). Menthofuran was found to have structural elements of a cytochrome P450 and most closely resembles a cytochrome P450 monoterpene hydroxylase (Lupien et al. 1995; Lupien et al. 1999; Bertea et al. 2001). From this it was theoretically expected and later proven, that the administration of peppermint oil has inhibitory effects on humans cytochrome P450, leading to an increase of bioavailability for drugs being substrates for cytochrome 450 (Dresser et al. 2002). P-glycoprotein mediated efflux also leads to an decrease in bioavailability, but can be revealed by the co-administration of peppermint oil (Wacher et al. 2002). Therefore the combination of peppermint oil and cyclosporine being a substrate for both, cytochrome P 450 and p-glycoprotein, might lead to formulations with an increased bioavailability of cyclosporine.

### 3.1.6 Materials for determination of the refractive index

#### 3.1.6.1 Fat emulsions

Lipofundin N 10% and Lipofundin MCT 10% (BBraun-Melsungen AG, Melsungen, Germany)

#### 3.1.6.2 SLN/NLC

Cetylpalmitate (Cutina CT) (Cognis Deutschland GmbH & Co. KG, Düsseldorf, Germany); Softisan 601 (Condea Chemie GmbH, Witten, Germany); stearyl alcohol (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany); Dynasan 116 (Condea Chemie GmbH, Witten, Germany); Miranol Ultra C32 (BASF AG, Ludwigshafen, Germany); Tyloxapol (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany); Tegocare 450 (Goldschmidt GmbH, Essen, Germany); liponic acid (Synopharm GmbH, Bursbüttel, Germany); tretinoine (HERMAL Kurt Herrmann GmbH & Co. OHG, Reinbek, Germany); α-Tocopherol (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany)

#### 3.1.6.3 Nanosuspensions

Azodicarbonamide (ADA) (Fluka Chemie AG, Neu-Ulm, Germany); budesonide (PharmaSol GmbH, Berlin, Germany); carbamazepine (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany); itraconazole (Technology Catalysts, Falls Church, USA); bethamethason-valerat (PharmaSol GmbH, Berlin, Germany); buparvaquone, (Innova Specialties Inc., Missouri, USA)

#### 3.1.6.4 Oils

linseed oil, poppy seed oil, peanut oil, corn oil, almond oil, olive oil, castor oil, saflor oil, soy bean oil, sun flower oil (Henry Lamotte GmbH, Bremen, Germany); fish oil from menhaden (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany); Migliol 812 (Caesar & Loretz GmbH, Hilden, Germany)

#### 3.1.6.5 Latex dispersions

Latex dispersion batch code: BMG 22 (concentration of particles =7.2 w%), latex dispersion batch code: 60/II (concentration of particles =4.45 w%) and latex dispersion batch code: SCA18 (concentration of particles =4.7 w%), were kindly provided from Dr. B. Paulke (Fraunhofer IAP, Potsdam-Golm, Germany); latex standards 0.102µm, 0.204µm, 0.404µm, 0.845µm and 2.875µm, were purchased from PostNova Analytik GmbH (München, Germany)

### 3.1.7 Other materials

Acetonitrile and isopropyl alcohole (Carl Roth GmbH, Karlsruhe, Germany); glycerol, sodium chloride, potassium chloride, aluminium chloride (Merck AG, Darmstadt, Germany); Sandimmun optoral (Novartis AG, Basel, Switzerland)

Purified water was obtained from MilliQ plus (Millpore, Schwalbach, Germany)

## *3.2 Methods*

### 3.2.1 High pressure homogenisation

#### 3.2.1.1 Nanosuspensions

All nanosuspensions were produced using an APV 40 (Niro Soavi, Lübeck, Germany) in its discontinuous production mode, yielding a batch size of 40ml per production. The production for all nanosuspensions of this thesis is principally the same. Special conditions used are explained separately in the corresponding chapters. Prior to homogenisation the dispersion medium is prepared by dissolving the stabilisers in the water phase. The prepared aqueous phase is then subsequently added to the drug placed in a mortar with pistill. This procedure is required for highly lipophilic drugs, as simple addition of the drug to the suspension medium causes agglomerates and the incorporation of air. The gentle preparation using mortar and pistil already destroys large agglomerates and avoids incorporation of air. Further diminution of larger crystals is then obtained by dispersing the suspension using an Ultra Turrax T25 (Jahnke & Kunkel, Staufen, Germany) for 3x30s at 9,500-15,000rpm. The resulting pre-mix is transferred into the bulk container of the high pressure homogeniser. Homogenisation is run with 3 cycles of 100, 250 and 500 bar in order to avoid blocking of the homogenisation gap and extraordinary erosion of the homogenisation valve caused by large crystals. After wards high pressure homogenisation is conducted at 1500bar for 40 cycles. The progress of homogenisation is controlled by characterising samples taken after cycle 1, 5, 10, 15, 20, 30 of the high pressure homogenisation procedure. Samples were characterised by light microscopy, PCS and LD measurements.

#### 3.2.1.2 Emulsions and lipid particles

The production of emulsions and lipid nanoparticles via high pressure homogenisation was also conducted using a discontinuous high pressure homogeniser APV40 (Niro Soavi, Lübeck, Germany). However pre-emulsification and homogenisation were performed at temperatures of 80°C. The lipid phase and the aqueous phase were heated separately prior unification. Afterwards pre-emulsification was conducted using an Ultra Turrax T25 (Jahnke & Kunkel, Staufen, Germany) for 60s at 9,500-15,000 rpm. The pre-emulsion was transferred

into the assembled homogeniser equipped with a heating jacket for temperature constancy and homogenised 3 times at 500 bar. In some experiments emulsions were not produced at elevated temperatures but at room temperature, which is mentioned in the respective chapters.

### 3.2.2 Photon correlation spectroscopy (PCS)

Photon correlation spectroscopy (PCS) was performed using an N4 plus (Beckman-Coulter, Krefeld, Germany) for the cyclosporine nanosuspensions. For all other systems investigated the Zetasizer 4 (Malvern Instruments, Herrenberg, Germany) was used. Further investigations were performed using the Nanosizer 4 (Malvern Instruments, Herrenberg, Germany). Measuring conditions were 20°C for the cyclosporine nanosuspensions and 25°C for all other systems investigated. All samples were analysed 10 times at an angle of 90° and with automatic sampling time mode. All nanosuspensions were analysed in deionised and water saturated with the drug. Latex particles, emulsions and SEDDS were analysed in deionised water. PCS also known as dynamic light scattering yields a light intensity weighted mean diameter (z-average) as a measure for the size of the bulk population and the polydispersity index (PI) as a measure for the with of the size distribution. The measuring range of these instruments is from 3nm to 3µm.

### 3.2.3 Laser diffractometry (LD)

The instrument used in this thesis was the LS 230 from Beckman-Coulter (Krefeld, Germany). Details of the method, as well as the applied parameters are explained in the main LD chapter of this thesis (chapter 4).

### 3.2.4 Microscopic analysis

A Leitz-Orthoplan (Wetzlar, Deutschland) was used. Samples were analysed (n=2-3) with 160 fold magnification to obtain an overview information of the whole system and at a magnification of 1000 (oil immersion) for detailed information. Polarised light was used for the detection of crystals at a magnification of 160 and 400. The Becke line was observed with magnifications appropriate for the respective system (40-1000x).

### 3.2.5 Zetapotential determination (ZP)

The zetapotential (ZP) was analysed also using the Zetasizer 4 (Malvern Instruments, Herrenberg, Germany). For this the zetasizer was switched from PCS mode to the laser doppler anemometry (LDA) mode. The measurement itself is a particle electrophoresis, the particle velocity is determined via the Doppler shift of the laser light scattered by the moving particles. The field strength applied was 20 V/cm. The electrophoretic mobility was converted to the zetapotential in mV using the Helmholtz-Smoluchowski equation. At standard

measuring conditions (room temperature of 25 °C, water) this equation can be simplified to the multiplication of the measured electrophoretic mobility (µm/cm per V/cm) by a factor of 12.8, this yields the ZP in mV. To study different aspects of the adsorption of ionic and non ionic stabilisers, different measuring media had to be applied. For the determination of the Stern potential measurements were performed in bidistilled water having its conductivity adjusted to 50 µS/cm. Using a standard conductivity adjusted with a 1:1 electrolyte avoids fluctuations in the zetapotential due to variations in the conductivity of distilled water which can range from approx. 1 up to 10 µS/cm, especially when the sample itself contains also electrolytes when adding it to the distilled water. For detailed explanations see (Müller 1996). Furthermore adsorption onto the drug nanocrystal surface was studied as a function of the stabiliser concentration, sodium chloride was used to adjust the thickness of the adsorbed layer (Müller 1991).

### 3.2.6 Measurement of the refractive index (RI)

The real refractive index was calculated from dn/dc measurements. The dn/dc, also called differential refractive index is the variation of the real refractive index due to a change in concentration. It is also known as the specific refractive index increment with the given symbol ν. It is expressed as g/ml. However, mostly it is called dn/dc.

$$\nu = \left.\frac{dn}{dc}\right|_{c=0} = \lim_{c\to 0}\left(\frac{n-n_0}{c}\right)$$

The measurement of dn/dc gives the possibility to calculate the index of refraction of an unknown compound. This is possible because the refractive index of a compound increases linearly with an increase in its concentration, when it is dissolved or diluted in another medium.

The procedure and the calculation of the refractive index is described in the literature (Huglin 1972; Wu and Xia 1994; Russo 2005). The procedure used was kindly overtaken from the workgroup of R. Sigel (Sigel).

Different dilutions with various concentrations (w/v) were prepared from the sample with the unknown refractive index. Afterwards the real index of refraction was measured for each sample prepared. In addition a sample of pure dilution medium was analysed. From the data obtained the differential index of refraction was analysed. For that the measured index of the pure dilution medium was subtracted from each index measured for the different concentrations. The obtained set of data was plotted in a diagram; where the concentration is plotted on the ordinate and the refractive index n is plotted on the abscissa. From this the linear regression function was calculated using Microsoft Office Excel calculations. The

obtained slope m of the function corresponds to the dn/dc in g/ml. In order to calculate the real refractive index of the unknown compound, the obtained differential refractive index was multiplied by 100, which corresponds to 100% of the compound. Finally the refractive index of the pure dilution medium was added and the value for the real refractive index obtained. Extraordinary modifications of the method are separately described in the main part of this thesis.

Measurements of cyclosporine, azodicarbonamide and carbamazepine nanosususpensions were conducted using a scanning Interferrometric Refractometer (NFT Scan Ref, (Nanofilm Technology, Göttingen, Germany) attached to SEC columns with a cuvette length of 2.142cm, operating at a laser wavelength of 632.8nm and 20°C. Raw data were processed using PSS-WinGPC V4.02 and NFT V2.0c software. All other particular systems were analysed using an automatic digital refractometer Abbemat-HP (Dr. Wolfgang Kernchen GmbH, Seelze, Germany). The instrument having a measuring range from 1.32-1.56 and a resolution of 0.000001 was operated at a wave length of 589.3nm (sodium line) and at 20.0°C. The accuracy of this highly sensitive instrument was 0.00002 for the refractive index and 0.03 °C for the temperature respectively. To avoid sedimentation of particles during the measurement a micro through stream cuvette ADK17 was used, each measurement was repeated 3-4 times. Some liquids, e.g. oils, were analysed by directly measuring the real refractive index by using a manual Abbe refractometer (Carl Zeiss, Jena, Germany), resolution 0.0002, and accuracy 0.001.

#### 3.2.6.1 Becke line

In addition for some of the samples the Becke line was observed. The Becke line is a band or a rim of light visible along a particle or crystal boundary in plane-polarised light. The method can be used to determine if a crystal or a particle has a lower or higher index of refraction than the surrounding medium. A Becke line is the result of two facts. Both are related to refraction along the boundaries of particles. First there is the fact that particles act as lenses as they tend to be thicker in the centre and thinner towards the edges. Therefore, if the real index of refraction is higher as the surrounding medium, the rays of the incident light converge towards the centre of the particle. If the index of refraction is lower than the surrounding medium the rays converge towards the edge of the particle. Internal reflection of the incident light occurs within the particle due to the presence of vertical particle boundaries. These two effects concentrate the light into a thin band within an object with a high index of refraction (Schmidt and Heidermanns 1958; El-Hinnawi 1966; Richardson 1991; Stroiber and Morse 1994; N.N. 1997; Derochette 2005). In Figure 3-3 this is illustrated for diamond dust and air

bubbles suspended in water. It clearly shows the bright ring around the diamond dust having a higher refractive index than water (refractive index diamond 2.41 ($d^{20}$), refractive index water 1.33 ($d^{20}$)). In contrast the air bubble having a smaller refractive index than water ($d^{20}$=1), here the bright ring is located inside. For the observation the light microscope Leitz–Ortophlan was used at appropriate magnifications. As references air bubbles in water (lower refractive index than the surrounding medium), latex particles and diamond dust (Vollstädt Diamant GmbH, Michendorf, Germany) (higher refractive index than the surrounding medium) were used. The references were always analysed in parallel to each measurement.

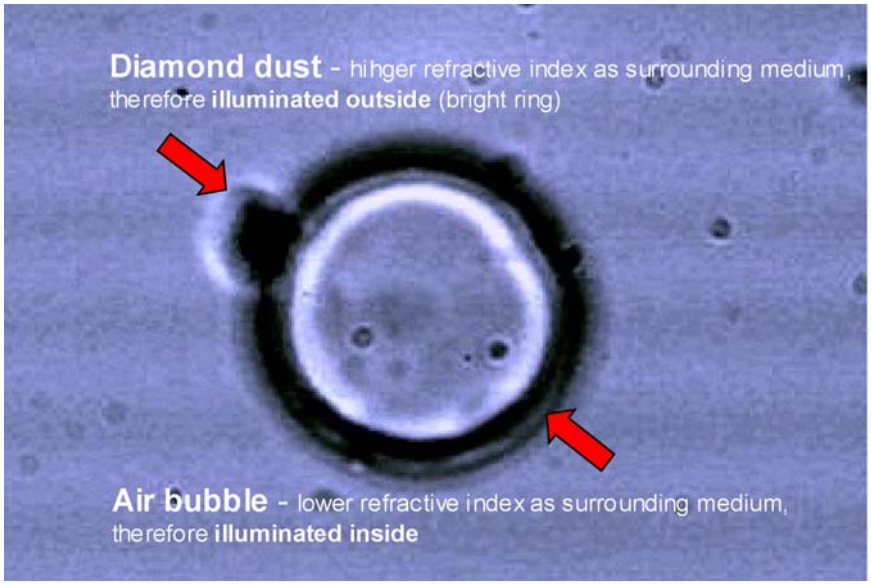


**Figure 3-3 Becke line for diamond dust and air bubble, particles with higher refractive index than the surronding medium are illuminated outside, whereas particles with lower refractive index are illuminated inside**

### 3.2.7 High performance liquid chromatography (HPLC)

The saturation concentration of cyclosporine and cyclosporine nanosuspensions was detemined by high performance liquid chromatography (Kontron Instruments, Neufahrn, Germany). The HPLC method for cyclosporine was used after having a detection limit of 2.2µg/ml and a precision of 3.5%. The mobile phase was produced from water, acetonitrile and isopropyl alcohol (20:70:10). The flow rate was set to be 1.2ml/min, the temperature of the oven was adjusted to 60°C and the UV detector was operated at 210nm. The columns used were RP18, 5 µm, LiChrospher as a pre-column and RP18, 4µm, 250 x 4mm, Superspher-100 as the main column. The injection volume was 20µl and running time was 12min. The retention time of cyclosporine under these conditions was 7.5min.

### 3.2.8 Differential scanning calorimetry (DSC)

Differential scanning calorimetry was performed in order to prove the absence of cyclosporine crystals in peppermint oil solutions absorbed onto Aeroperls® 300. For that a computer interfaced differential scanning calorimeter DSC 821e (Mettler Toledo AG, Gießen, Germany) with the Star software Version 6.0 was used. Samples were weighted accurately and heated from 0°C (25 °C) to 180 °C with a heating rate of 10K/min.

### 3.2.9 UV/Vis measurements

UV-Vis measurements were performed using the Kontron Uvikon 940 two beam spectrometer (Kontron Instruments, Neufahrn, Germany).

### 3.2.10 Surface tension measurements

The surface tension measurements were performed with a Krüss Digital-Tensiometer K 10 using the ring method.

### 3.2.11 Production of *s*elf-*e*mulsifying *d*rug *d*elivery *s*ystems (SEDDS)

For producing the SEDDS, the pre-concentrate (lipophilic phase, emulsifier) was mixed and stirred for 10min, to ensure homogeneity. Water was added rapidly using a 10.0ml pipette. After adding the water, the obtained system was stirred using a magnetic stirrer (oblong 6mmx 3.5mm). Time and speed were controlled. Cyclosporine used was dissolved prior usage in peppermint oil. This concentrate, containing 33% (w/w) of cyclosporine was diluted if required for the different formulations investigated. All systems obtained were analysed by light microscopy and photon correlation spectroscopy. Appropriate samples were chosen and analysed by laser diffractometry.