Investigation of Nanoparticulate Formulation Intended for Caffeine Delivery to Hair Follicles

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Dekan : Prof. Dr. Volkhard Helms

Berichterstatter : Prof. Dr. Marc Schneider (Universität des Saarlandes)
                 Prof. Dr. Alexandra Kiemer (Universität des Saarlandes)

Vorsitz : Prof. Dr. Gregor Jung (Universität des Saarlandes)

Akad. Mitarbeiter : Dr. Maike Windbergs (Universität des Saarlandes)
Die vorliegende Dissertation entstand unter der Betreuung von

Prof. Dr. Marc Schneider

in der Fachrichtung Biopharmazie und Pharmazeutische Technologie
Arbeitsgruppe Pharmazeutische Nanotechnologie
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SHORT SUMMARY

Caffeine in cosmetic products such as shampoo and lotion can stimulate hair growth. Recently, it was shown that nanoparticulate formulations have better penetration into hair follicles. Moreover such particulate formulations to stimulate hair growth are not available commercially. Therefore in this study, two systems have been developed. The first was a particulate formulation loading caffeine by utilizing biocompatible and biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) and chitosan. The second was a model of hair shafts containing nanoparticles (NPs) to prove that such model hair follicles can be visualized by photoacoustic microscopy. Results revealed that there is an interaction between chitosan and caffeine suitable for drug loading. Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and solubilization showed that this interaction is based on complex formation. NPs characterization showed that NPs have a spheric shape. NPs from chitosan-PLGA showed good properties in terms of particle size and distribution. Loading of caffeine was increased by using chitosan up to 19% EE. Caffeine release from NPs is slower than from the pure complex allowing a longer time frame for continuous drug release. For imaging of NPs in model hair follicle (voids in agarose gel), PLGA NPs loading magnetite were prepared. These NPs could be successfully imaged by photoacoustic microscopy inside the model hair follicle.
1.1 Hair loss problem in human life

Hair loss can happen to men and women. Patients are usually afraid of experiencing it, because hair loss can lead to baldness [1]. The influencing factors causing hair loss are shortly reviewed in terms of the conditions of the hair in the follicles and the biosynthesis of hair which impact to the fragility of hair. Testosterone and dihydrotestosterone are androgenic hormones which are involved on hair loss. Overall, hair loss is influenced by internal factors which ranging from enzymes to hormones and external factors [2-5].

1.1.1 Internal and external factors causing hair loss

Internal factors involve genetic reasons controlling the metabolism of hormones and active substances relevant for hair growth. The gene \( \text{Fgf5} \) is responsible to encode the transcription factor for expression of fibroblast growth factor 5 (FGF5). This growth factor plays an important role in the proliferation of hair follicles. Three types common of baldness are due to genetic reasons: The first is \textit{androgenic alopecia} which can be seen anytime after puberty [6,7]. Usually the problem will increase by increasing age. The second is \textit{telogen affluvium} which is often associated with genetics and hair loss occurs especially due to many hair follicles suddenly stopping growing because of hormone imbalance (e.g., pregnancy) [6,8,9]. Both these types of baldness happen in sequence until all hair is lost. The third type is \textit{alopecia areata} as shown in Fig.1 which indicates the spot or patchy baldness [10,11].

Other internal factors were thyroid imbalance, allergic reactions and diabetes mellitus. When \textit{thyroid imbalance} occurred, high or low thyroid concentration can interrupt the biosynthesis of testosterone and dihydrotestosterone that influences the hair growth. Hair loss happened when testosterone is converted to dihydrotestosterone [12]. \textit{Allergic reaction} as the manifestation of hypersensitivity reaction impacts on the activation of progressive fibrosis of the perifollicular sheet occurs in lesions [13,14]. \textit{Diabetes mellitus} can also cause hair loss. The growth of hair is influenced by the concentration of glucose which can interact with keratin of hair follicle which is known as glycosylation. Diabetes also influence the blood
circulation to hair follicles affecting the ability of the hair follicle to form metabolites [15-18]. This also reduces the likelyhand of hair growth due to malnutrition [15-17].

Figure 1. Patchy hair loss “common type of alopecia” (image included with permission of volunteer)

External factors are the factors out of the human body such as substances and environmental conditions which impact on the human body. External factors involve psychic disorder, consumption of drugs such as chemotherapeutics and such simple issues as hair styling. Psychic disorder because of stressful daily life can also lead to several diseases including hair loss. Many works in modern life style lead to less spare time for cooling down the metabolic reaction of the body. To support these activities, the body also needs good nutrition for respective metabolic action. Lack of protein and vitamin intake can cause hair loss. Therefore consuming sufficient nutrition is necessary. Especially vitamins of vegetables and fruits play a role in supporting the strength of hair root [19]. Consumption of anti cancer drugs such as derivatives of cisplastin, aclarubicine and doxorubicine reduce the proliferation of hair cells by being toxic especially to proliferating cells [20-22]. Hair styling is another factor which impacts on the growth of hair especially when chemicals and mechanical equipment are involved [23].

1.1.2 Prevention and treatment of hair loss

The natural way to prevent hair loss is to make it become healthy. Low hair vitalization and dandruff formation can increase the probability of hair loss. All efforts in order to maintain vitalization and to diminish dandruff formation are traditional way that people applied since long time ago [24,25].

The treatment of hair loss is typically connected with an effort to reduce the concentration of dihydrotestosterone [26]. On stem cells, there are receptors which can bind testosterone. Afterwards, stem cells in the presence of testosterone [27] induce the production of specific ligands interacting with natural killer cells (NK cells). The ligands are proteins of stem cells also activating monocytes which are then
involved in hypersensitivity and cause inflammation as trigger for the degradation of the root of hair follicle. As consequence, hair follicles can not grow [28]. The condition when the hair follicle can not grow is known as miniaturization of the hair follicle. At the basal layer of hair follicles the dermal papillae is located. It contains fibroblasts which can regulate hair growth. The fibroblasts have androgen receptors. The larger sensory nerve branches and the blood vessels that nourish the skin are also located in the dermal papilae. This layer is an important part to inhibit the change of testosterone to dihydrotestosterone by using active substances such as caffeine [29,30]. Based on this information, the strategy to treat the hair loss is by inhibiting the conversion of testosterone to dihydrotestosterone. For this purpose, caffeine and also estradiol have been used [26,31]. Treatment of hair loss has also already been conducted by using commercial products such as lotions, creams, hair sprays and shampoos [32].

1.1.3 Caffeine as stimulating agent to stimulate hair growth

Rubiaceae plants are used in pharmaceutical application since long time ago as well as in beverages. These plants have several bioactive compounds such as caffeine which belongs to the alkaloid purine. As an alkaloid, Caffeine is obtained in varying quantities in beans, leaves and fruits of plants such as coffee, tea, cacao, and cola [33]. The presence of caffeine in those plants is to defend against pathogens. People use the leaves of tea (Fig.2) for common consumption as well as coffee, cola and cocoa bean. These plants are also used in commercial products. These products are legally unregulated in nearly all jurisdictions even though containing psychoactive substances [34,35].

Figure 2. Camellia sinensis is well known as tea plant. Image was taken in private garden in Bandung, Indonesia.
Caffeine consumption improves the physical endurance towards reduced drowsiness and restoring alertness, cognitive function, particularly vigilance, mood and perception of fatigue [25,33].

Caffeine is a white powder with a hexagonal crystal structure of alkaloid purine. Caffeine has a molecular weight of \( M_w = 194.19 \text{ Da} \) and the monohydrate has the \( M_w = 212.12 \text{ Da} \). The melting point of caffeine is \( 238^\circ C \). As can be concluded from \( M_w \) which was sketched in Fig.3 the chemical name is \( 1H\text{-purine-2,6-dione, 3,7-dihydro-1,3,7-trimethylpurine} (\text{C}_8\text{H}_{10}\text{N}_4\text{O}_2) \) with C 49.48%, H 5.19%, N 28.85%, O 16.48% [36].

![Molecular structure of caffeine](image)

Figure 3. Molecular structure of caffeine

As a central nervous system (CNS) stimulant, caffeine can increase brain activity by blocking the receptor for neurotransmitters such as dopamine, serotonin, acetylcholine, glutamate and \( \gamma \)-aminobutyric acid. Regarding hair growth, caffeine does not act directly to the main pathway of hair synthesis. However, caffeine and cAMP have a similar structure. Under physiologic condition, the concentration of cAMP is decreased by dephosphorylation reaction. In the presence of caffeine, dephosphorylation can be inhibited and therefore the concentration of cAMP is increased [31,37].

1.2 Topical and follicular nanoscale drug delivery systems

The barrier function of human skin imposes physicochemical limitations to the permeation of drugs that can cross this barrier. For a drug to be delivered passively via the skin is difficult. An adequate lipophilicity is necessary to enhance the permeation [38,39]. However, a strategy has been developed to direct delivery based on particulate formulations to hair follicles [40]. This application has an advantage for an implementation of topical and follicular drug delivery systems. The investigations
have been conducted to evaluate this new, innovative, and convenient dosage form to target hair follicles [41].

1.2.1 Topical nanoscale drug delivery systems

Topical drug delivery systems are systems used to mainly apply drugs on the skin to obtain localized effects at the site of application. Topical drug administration is supported by the ease of administration and need to take into consideration the skin structure as described in the following. Skin as the largest organ, has an area of 1.7 m$^2$ and approximately 4 kg in weight or about 5.5% of the body mass [41,42].

![Figure 4](image)

Figure 4. A schematic sectional view of skin which involves stratum corneum (SC), viable epidermis and dermis (Image is adapted from [43])

Skin is known as the outer barrier between the body and the environment and protects the body from external chemicals and pathogens. Skin is made up of three cellular layers as is shown in Fig.4. Each of them has its own structure and function. The outermost cellular layer of the skin is the epidermis which is composed of the viable epidermis consisting of living cells and the non-viable stratum corneum (SC): cornified cells forming a densely packed layer being the strongest barrier of the skin. The dermis lies directly underneath the epidermis and consists of compact connective tissue nerded with blood and lymph vessels. This formation supplies the epidermis with nutrients and removes absorbed exogenous substances acting as sink. The subcutaneous fatty tissue in dermis as well as other skin layers, consist of loose connective tissues, and its dimensions vary greatly. Topical drug administration
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gained attention as the skin offers an easy and hence convenient route having great potentials to deliver drugs compared to other drug administration such oral, rectal and parenteral [44].

Interestingly, for topical application, nanoparticles (NPs) are already used in cosmetics [45]. Talking about terminology of nanoparticle, nano means small originating from the Greek term for dwarf. Materials which have dimensions between 1 and 100 nm can possibly show unique properties enabling novel applications. Nanotechnology is the creation or use of the nanometer-sized materials [46,47]. In this thesis the usage of nano’ will be extended with respect to nanomedicines where also sub-micron ranges are considered to be part of it.

Although NPs in cosmetics are commercially available, it is still under debate if penetration into the skin is happening and to which extend. Nevertheless, several results of investigation about penetration NPs across the skin were reviewed [48-50]. Several studies investigated the penetration of inorganic NPs such as gold NPs [51], lipid-based NPs [52] such as liposomes [53], transferosomes [54], ethiosomes [55], solid lipid nanoparticles (SLNs) [56], nanostructured lipid carriers and surfactant based systems such as nanosomes [57], micelles [58], and nanoemulsion [59].

Based on the investigations regarding the penetration of NPs into the skin, 6 nm AuNPs showed much higher extent than 15 nm AuNPs. Furthermore, it indicated a minimal effect of the vehicle on particle penetration [51,60,61]. The investigation of topical application of Fluorescein-PLGA NPs with size 320 nm reported that particles were only distributed on the surface of the skin. The measurement was conducted by CLSM and revealed that particles located near to the lipid layer around the corneocytes [62]. In conclusion, only very small particles seem to be able to successfully penetrate into the deeper skin layers. The determination of the amount of NPs in the SC could be done by tape-stripping [62,63]. Furthermore, the experimental set up for a penetration experiment for NPs were also studied. For instance, exposure times of at least more than 6 hours were recommended for future studies on skin penetration of NPs. These obtained informations are very important for the basic understanding of the interaction of NPs with the skin barrier [51,64]. This would be of use for future pharmaceutical and clinical applications, e.g. designing optimal topical and transdermal delivery systems. Additionally, some drugs have been evaluated to be delivered into the skin; for instance the evaluation of anti aging, vaccine and anti malignant melanoma [65-67].
1.2.2 Penetration pathways across the skin

Substances delivered into the skin involve three major pathways as shown in Fig.5. The possible routes are transcellular, intercellular and transfollicular pathway. The transcellular pathway is considered to be of minor importance for dermal absorption due to low permeability of certain substances through the corneocytes [68]. In this transport pathway, substances have to partition from hydrophilic corneocytes to the lipid layers of the SC repeatedly resulting in a very slow process [69]. The intercellular pathway is considered as the predominant pathway for most substances. In this case, substances diffuse within the continuous intercellular lipid domains of SC [70,71]. In contrast to the corneocyte structure which is compact, the lipid domain pathway can absorb the substances faster than corneocytes and also a higher amount of the substances can be absorbed. Furthermore, this pathway can be an alternative especially in the presence of penetration enhancers [51]. It is known that the permeability of corneocytes increases due to alteration of keratin structure. However, nowadays the transcellular pathway has been investigated and may be more relevant to evaluate new formulations for dermal therapy [72].

As obvious from Fig.5 the third pathway is the skin appendages offering a direct passage into deeper skin layers. A high density of blood vessels around the hair root and the absence of the SC make this an intriguing pathway especially for
the hair follicles also showing the possibility to be permeated by drugs [74-77]. Some research was focused on the evaluation transfollicular drug delivery [78]. More detailed explanations about transfollicular pathway will be given in the next chapter 1.2.3.

1.2.3 Transfollicular pathway for drug delivery systems

Transfollicular pathway can deliver drug substances and particles into hair follicles. A respective image of a hair follicle is shown in Fig.6. Hair follicles are embedded in the epidermis extending deep into the dermis which provides a much greater actual area for potential absorption below the skin surface.

The structure of the hair follicles is described as a complex structure which is formed by three common parts: dermal papilae, bulge and sebaceous, and also the infundibulum [79]. Each part represents the distinct program of differentiation for follicle morphogenesis during the embryonic cycle. The dermal papilae in basal layer is the most important layer. This layer is covered by the follicular epithelium. The dermal papillae contains specialized cells, so-called fibroblasts, that regulate hair growth. Above this layer, are located the bulge area and the sebaceous glands [80]. The next part is the infundibulum. From recent evidence about regulation of hair growth is known that the infundibulum area also has a function to regulate follicular growth and differentiation [81]. The location of bulge, sebaceous gland and infundibulum are schematically shown in Fig.7.

Cosmetic products have been applied on the surface of skin but pharmaceutical dosage forms are facing the problem to deliver drug into the skin. To bring the drug across the stratum corneum is not an easy task. However, hair follicles
give a significant contribution to the penetration which is known as transfollicular pathway [82,83]. In the past decade there was not much attention to this pathway [84]. A study about non particulate formulation was done to investigate its ability to penetrate hair follicles. The substance chosen for this study was estradiol. It is a poorly soluble, neutral compound with log octanol-water partition coefficient of logP = 2.29 and a water solubility of 0.0003%. In 2002 the sandwich model with SC membranes for a Franz diffusion cell experiment was described. By using this SC sandwich, putting two sheets of SC on top of each other, there is only a negligible chance for a direct connection of two openings (hair follicles) across both membranes because of the random distribution of hair follicles on the skin surface. The result showed that the permeation through the sandwich was much reduced rather than that of a single skin membrane (SC) [85].

The group of Jürgen Lademann at the Centre for Experimental and Applied Cutaneous Physiology, Charite University of Medicine Berlin, Germany, investigated the delivery of cucurmine as substance for topical application. The presence of cucurmine was determined by using confocal laser scanning microscopy (CLSM) of skin biopsies. It could be shown that the substance could penetrate by using follicular pathway [83].
Furthermore, the transfollicular pathway was found to be accessible for sub-
micron particles. Hence these systems can be used as drug delivery systems to
specifically target the hair follicles. This specificity might turn the nano- and sub-
micron carriers into important drug delivery system for the skin. A study about the
penetration of TiO$_2$ microparticles contained in sunscreens was conducted to
determine their ability to penetrate hair follicles by using tape-stripping as well as by
using X-ray fluorescence microscopy. It could be shown that these particles
penetrated into the hair follicles [87]. Further investigations have even demonstrated
that NPs have a better penetration than non-particulate formulations[80]. Regarding
the particles size, microparticles of size 3–6 µm showed a tendency to aggregate in
the hair follicles [88,89]. Particles in the size of 750 nm showed a homogenous
distribution in the hair follicles [88]. Lademann reported in 2009 that particles sized
300–600 nm penetrate efficiently into hair follicles than larger particles [90]. This size
range corresponds to the approximate size of the hair cuticula which is 530 nm for
human hair and 320 nm for pig hair (which is assumed to play an essential role in the
permeability process by a pumping process due to the movement of the hair) [90].

Also particles of ~100 nm in size were found to be able to penetrate into the
hair follicles after sunscreen application [86]. Even smaller particles with sizes as
small as 40 nm were found to penetrated deeper into the follicle and could also reach
the follicular epithelium [91]. These small particles allow also to penetrate in the
sourrounding tissue. According to the definition of NPs as particles being around 1–
100 nm it can be concluded that NPs are better than microparticles to address
cellular internalization around the hair root for instance in langerhans cells for
possible vaccination [92].

The underlying mechanism for the superior particle penetration was explained
to be due to the hair working as a geared pump in the hair follicle area. Hence the
movement of the hair drives the particles deep along the hair into the hair follicle. On
the other hand, the investigations conducted by Lademan in 2006 and Otberg in
2007 revealed that hair follicles also represent an efficient storage for long term
which keep those particles in reservoir [62,80].
1.3 Particulate formulation as drug delivery systems

Nanotechnology has found applications in nearly all fields leading to significant technological advantages that can be applied such as in the pharmaceutical field for preparing and characterizing nanoparticulate formulations. Particulate formulations are stable products and they have large potential to target and control the release of encapsulated drugs for instance due to different ways of interaction. Many efforts are focused on the development of these particulate formulations [93].

1.3.1 Current status of polymeric nanoparticles

Polymeric nanoparticles (NPs) are NPs which were prepared by using polymers. For pharmaceutical application, biodegradable and biocompatible polymers are preferred. The advantages to use such polymers are because of that the degradation of these polymers to biologically acceptable molecules that are metabolized and removed from the body using normal metabolic pathways. These biodegradable polymers also are well known as safe and bio-tolerant because their products of degradation and by-products fulfill the request for little or no adverse reactions within the physiology of the human body. The success of loading polymeric NPs with drugs shows that nanotechnology can provide carrier systems which can and will be used for many exciting products potentially overcoming many hurdles in formulation technology [94].

The drug is entrapped, encapsulated or attached to the NPs. Depending on the method of NP preparation, nanospheres or nanocapsules can be obtained [95]. In recent years, biodegradable polymeric NPs based on natural polymers such as chitosan, gelatin, alginate or synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA), poly (anhydrides), poly (caprolactone), poly (ortho esters), and poly (amino acids) have attracted considerable attention as potential drug delivery systems in view of their applications in drug targeting especially to particular organs/tissues [96,97]. Among these polymers, PLGA is most often used and was already used in implant medical products [98].

Some researchers used PLGA in laboratory scale to form NPs and it is due to its benign nature a promising material to be used in future pharmaceutical products. As can be seen from the structure, PLGA is condensed by a lactic acid block and glycolic acid block by ester bonds forming a block copolymer (Fig.8).
The molecular weight of PLGA ranges from 5,000 to 50,000 depending on the composition of lactic and glycolic acid blocks. The higher the number of lactic and glycolic acid blocks or the respective lactide block, the bigger is the polymer. PLGA is a semipolar polymer which dissolves in semipolar solvents such as ethyl acetate, acetone, and dichloromethane. For the preparation of nanoparticles [96,99], PLGA is often dissolved in ethyl acetate as an organic phase and the stabilizer is added into the aqueous phase [96].

For investigation of drug delivery, particles based on poly lactic acid derivatives such as PLGA are used. These particles could be appreciable also for the delivery both of hydrophilic and hydrophobic drugs. However, in the past it was observed that hydrophobic drug can be loaded into the polymeric NPs very well. However, for hydrophilic drugs, the situation is more complicated and a double emulsion method is preferred to incorporate hydrophilic compartments containing the drug in the particles [100]. Another method such as nanoprecipitation is also used for hydrophilic drugs [101,102]. As possible hydrophilic pharmaceutical active agents, therapeutic proteins and vaccines were used. Until today, there are still limited data concerning PLGA NPs containing hydrophilic substances [103]. Looking in the literature, it turns out that PLGA is often applied as a drug carrier system to study the delivery and targeting of colon cancer drugs. Nevertheless, its biodegradability makes it also a useful material for other application routes. The PLGA NP formulations prepared are characterized in terms of size, dispersity index, zeta potential, and release profile [104]. The adjustment of these physical properties is the key for targeting as they correspond to the interaction with the target location of the NPs. The loaded hydrophilic drugs which were studied ranged from antibiotics, anti cancer drug, anti inflammation drugs, and therapeutic proteins including vaccines [48,105,106].

Besides PLGA, chitosan, gelatin and alginate are also relevant and important substances to form polymeric NPs. These polymers were grouped as natural
polymers. In contrast to the polymeric NPs based on synthetic polymers the natural polymers show a broad size distribution. Hence for basic understanding and investigation they are less suited and particles from synthetic materials such as PLGA are often preferred due to their better size distribution [107,108].

1.3.2 Preparation of polymeric nanoparticles

The PLGA NPs are appropriate for drug delivery systems and have been applied because of its biodegradability ensuring that the carrier itself and its products do not disturb the physiologic conditions of the human body. This pharmaceutical important ability resulted already in a pharmaceutical product containing PLGA, which is being approved by national food and drug administration (FDA) [98] hence underlining the materials' potential. Therefore, these NPs are used as vehicles for the targeted and controlled delivery of drugs. Investigations showed successfully that PLGA NPs are appropriate for various routes drug administration [96]. To form these NPs methods know from nanotechnology are used. This technology is appropriate to form NPs and also showed the ability to load during the process pharmaceutical substances in the particles. NPs based on PLGA of different physical characteristics such as size, distribution of particles, morphology and zeta potential can be synthesized by controlling the specific parameters of the synthesis.

To load hydrophilic drugs is more difficult than hydrophobic drugs due to miscibility problems between the drug and the particles' material. Nevertheless, Barichelo et al. in 1999 intended to evaluate the loading of hydrophilic drugs (insulin and valproic acid) in NPs which were formed by nanoprecipitation [102]. Another hydrophilic drug such as procaine hydrochloride [103] was shown to be successfully loaded into NPs.

It is known that the synthesis on NPs by available methods such as emulsion solvent diffusion could be used as common method for preparation of NPs based on PLGA. As shown in Fig.9 two phases -organic and aqueous- phase (which are partially miscible) are involved. Typically, the polymers are soluble in organic solvent and the surfactant as stabilizer is soluble in water as the aqueous phase. The organic phase is dropped slowly into the aqueous phase under stirring condition. A pre-emulsion is achieved after continuous stirring for 1 h. The size reduction of the preemulsion the so-called nanoemulsification is conducted by transferring energy into the system using mechanical processes such as ultraturrax (rotor-stator principle) or
ultrasonifier from 1 minute up to 5 minutes depending on the expected size of the particles (Fig.9). Dilution with water allows the organic solvent to diffuse into and mix with water. As a result a solid particle from polymer without solvent is formed. After that overnight evaporation with low vapor pressure is needed to remove the organic solvent form the surrounding.

The double emulsion method is a common method to address the solubility issues between polymer and drug and hence to encapsulate hydrophilic drugs. Similar to the single emulsion solvent diffusion, but in this method two emulsification steps are involved by using two kinds of stabilizers. Here the inner, first emulsion contains the drug in an aqueous environment surrounded by polymeric material which is then incorporated into another, larger water droplet. The dilution with water is also required to extract the organic solvent overnight.

Another method to prepare polymeric NPs is nanoprecipitation which was also applied for hydrophilic drugs such as therapeutic proteins [102,109]. Overall the method is similar to the solvent diffusion method, but for this method miscible solvents are needed instead of partially soluble solvents. The polymer is dissolved in the organic solvent such as dimethyl sulfoxide (DMSO), ethanol or aceton. Stabilizer is dissolved in aqueous phase.

The respective drug is placed in the solvent where it dissolves. When mixing the organic phase containing the polymer with the stabilizer-containing aqueous phase, the miscibility of the solvents leads to an immediate precipitation of the polymer. As the polymer-solvent diffuses away, the polymer collapsed and precipitates in the nano size because it is not soluble in water [100,102,103].
1.3.3 **Chitosan and chitosan-coated PLGA NPs**

Chitosan-coated PLGA NPs are formed by using chitosan and PLGA. The coating involves positive charge of chitosan and negative charge of PLGA to mediate the interactions [110,111]. Chitosan is a hydrolyzed extract of chitin from crustaceans’ hardshell, such as shrimp, crabs, insects, and also mushrooms. To obtain chitosan the main processes which are involved are the following: first, the skin of shrimp or crab was washed to be deproteinated. Then the sample is washed with acid solution to remove the lime as demineralization process. After demineralization, the acetyl groups of chitin were cleaved leading to chitosan. The ratio of deacetylation corresponds to the positive charge coming from primary amine groups of chitosan [112].

Chitosan is poly-D-glucosamine which is composed by more than 5000 units of monomers (glucosamine and acetylglucosamine) with a molecular weight up to 500 kDa (Fig.10). The monomer number from which chitosan is composed is not less than 16 [107]. For biomedical applications, chitosan is already used as wound dressing to stop bleeding and is in addition applied due to its antibacterial properties.
Chitosan (chit) is generally soluble in acidic solution due to the protonation of the amine groups. However, chitosan chloride usually can also be dissolved easily in water of neutral pH. Therefore, for the preparation of chitosan-coated PLGA NPs, chitosan was added into the aqueous phase and with a simple one step emulsion solvent diffusion, chit-PLGA NPs can be prepared [113]. These chit-PLGA NPs have also been used for investigation regarding transfection of antisense oligonucleotides and gene delivery due to their positive surface charge [110,114]. Besides for gene therapy, chitosan NPs have shown a good performance to load other drug for example rifampicin as antituberculosis agent [115].

1.3.4 Magnetite loaded polymeric NPs

The importance of nanotechnology has offered many opportunities in various research fields. Based on imaging and therapy, particularly inorganic NPs have received great attention because of their outstanding properties. Metal NPs have many advantages over small conventional molecules that include high molar extinction coefficient, high resistance to photo-degradation, size/shape dependent and tunable absorbance/scattering properties, which can be useful for imaging and therapeutic approaches. Especially the size/shape dependent and tunable absorbance/scattering properties can enable on-demand design for imaging or characterization purposes of many inorganic NPs such as magnetite [116].

Magnetite is iron oxide (Fe$_3$O$_4$) with superparamagnetic properties which render interesting properties as important molecule than can absorb the near infrared light and could be attracted with magnetic field. Paramagnetism is a form of magnetism where certain materials are attracted by an externally applied magnetic field. Paramagnetic properties are due to the presence of some unpaired electrons and form the realignment of the electron orientation caused by the external magnetic field. On the other hand, superparamagnetism is a form of magnetism which appears...
in small nanoparticles for instance magnetite particles. Their size is small enough that only one magnetic domain exists per particles. Therefore the magnetization can randomly flip direction at room temperature due to the thermal energy. When an external magnetic field is applied, their magnetic moments are aligned along the applied field. The interest in magnetic materials is due to their function to develop and serve in modern technology [99,116]. Magnetite nanoparticles (MNPs) which possess paramagnetic properties were already evaluated in the clinic as contrast agent [117] or as therapeutic option in glioblastoma [118,119]. Furthermore, the ability to direct and hence target these particles by local magnetic fields offers further potential therapeutic applications [120,121].

Magnetite was also incorporated in polymer particles. In this case, the magnetic moment of each magnetite will be able to rotate randomly in reference to the orientation of the MNP. The important property for biomedical application was the lack of magnetization after the colloid got stable to avoid the agglomeration [122].

The investigation which were conducted by Maity, 2007 and Dresco, 1999 presented the best compromise among appropriate magnetic properties such as saturation magnetization, stability under oxidizing conditions and safety for biological application [123,124]. The US Food and Drug Administration (FDA) has already approved the medical product such as Feridex® and Resovist®-containing magnetic NPs which was formed by a mixture of Fe^{2+} and Fe^{3+} [125].
II. Aim of Dissertation and Experimental Design

The problem of hair loss resulting in baldness is a serious health problem. So far there are cosmetic products as non particulate formulations such as shampoo and lotion containing caffeine to stimulate hair growth. In addition, cleansing creams and lotions containing chitosan intended to clean the skin are also available on the market.

Even though particulate formulations were shown to penetrate better into the hair shafts (the target for caffeine delivery) no particulate caffeine formulation was described so far for such a purpose. As consequence, no cosmetic product is based on particles for caffeine delivery. Therefore, this research was intended to develop a particulate formulation using the natural polymer chitosan and the synthetic polymer PLGA to form chitosan-coated PLGA NPs as a carrier system loading caffeine.

Hair follicles as potential target for those carrier systems require also the imaging of particles which penetrate into the hair follicles. Therefore, the formulation of nanoparticles which can be imaged in respective structures would be necessary. To address the problems with respect to the size of the hair follicles for imaging, a new approach based on photoacoustic microscopy was aimed. The follicular imaging with photoacoustic microscopy needs the right marker for particle visualization and therefore a drug carrier system containing magnetite was the next goal.

Before going to the rather complex in vivo systems (animal or human skin), the ability to image the particles at all in hair follicles was in focus. Therefore a system was developed to mimic hair follicles and to image the particles in this structure ex vivo.

Besides the overall direction of the thesis a focus on the experimental approach of the different chapters is described below: As loading of the nanoparticulate formulation was tried to be accomplished based on specific interactions between the drug and the carrier. This study investigates the
interaction between chitosan and caffeine by using FTIR and DSC measurement in combination with a solubilization study.

√ It was aimed to see whether the interaction pattern involved covalent bonds or rather a complex formation.

√ Once known the interaction pattern, the following work was aimed to know the binding capacity of chitosan which interacted with caffeine by using solubilization study.

After identifying the basic interaction parameters preparation and characterization of chitosan-PLGA NPs, it was in focus to find a good formulation.

√ The most common steps to make NPs are intended to be loading hydrophilic drug such as caffeine using emulsion solvent diffusion. As an organic phase has used PLGA which was dissolved in ethyl acetate and aqueous phase was chitosan and PVA solution. For control, double emulsion technique as a common method to encapsulate hydrophilic drug was applied.

√ This further study also intended to characterize NPs using zeta sizer, SEM and AFM.

√ The amount of caffeine in NPs was determined indirectly from the supernatant of suspension and directly by dissolving NPs. The release profile of caffeine from chitosan-PLGA NPs across a membrane was determined and compared to the complex alone and caffeine diffusion across a membrane.

As conventional technique, such as light microscopy requires sectioning of the sample. Another imaging approach such as photoacoustic microscopy was in focus. With this approach, a material which can enhance the contrast such as magnetite is needed. Therefore the third part of the thesis was thought to develop a particulate formulation containing magnetite particles for improved contrast. This formulation would be applied to image the model hair follicle. As mentioned above, this model is necessary to establish rather than working with the complex systems of \textit{in vivo} measurements. The workpackages for this part were:

√ Preparation of PLGA NPs loading magnetite using two kinds of magnetite. First one was magnetite which stabilized with aminohexanamine and the second one was magnetite was stabilized with oleic acid. Single emulsion solvent disffusion
method was applied to the preparation of NPs. For the comparison the nanoprecipitation method was used.

✓ Characterization of PLGA NPs loading magnetite in terms of size, dispersity index of particles, zeta potential, and morphology. The presence and successful loading of the core of magnetite was determined by AFM and TEM.

✓ Development of the model hair shaft and imaging by using photoacoustic microscopy. Model of hair shaft was based on agarose. The idea was to create voids in the size of human hair follicles. The model hair shaft should be filled with suspension of PLGA NPs loading magnetite for imaging. The imaging measurement was conducted by using photoacoustic microscopy in cooperation with IBMT, St. Ingbert.
III. Interaction of Chitosan and Caffeine

3.1 Introduction

Several efforts were addressed to the development of cosmetic formulations especially when using chitosan and caffeine [126,127]. Chitosan which is commercially available in formulations is of high-grade quality made from shrimp shell by deacetylation (75-90%). Furthermore due to the positive charge, chitosan can interact with DNA and therapeutic proteins to avoid degradation and facilitate cellular uptake [128-130]. Chitosan was also used successfully to form particles containing theophylline derived from xanthine (alkaloid) as a lead structure [131,132]. However, there were no data regarding the interaction of chitosan and caffeine.

Based on the molecular structure of chitosan and caffeine, they both have several polar functional groups which lead to hydrophilic properties. Usually polar interaction of the drugs could be categorized into dipole-dipole (Keesom) and dipole-induced dipole interaction (Debye) which involve dipole orientation of molecules with polarizable dipole moments. Depending on the structure of the molecule, both types of interactions can take place in the underlying van-der-Waals (vdW) forces [133,134].

To study the interaction between chitosan and caffeine, the information about their polarity is necessary. Their polarity refers to a separation of electric charges leading to a molecule or its chemical groups having an electric dipole or multipole moment. Polar molecules interact through dipole-dipole intermolecular forces and hydrogen bonds and molecule polarity depends on the difference in electronegativity between atoms in a compound and the asymmetry of the compound’s structure. Furthermore, polarity of the molecules results in a number of physical properties including surface tension, solubility, boiling and melting points [135,136]. This polarity usually corresponds to hydrogen bonds forming between the hydrogen atom attached to an electronegative atom such as oxygen in the carbonyl functional group (C=O) of caffeine, and nitrogen in the amine group N-H of chitosan. Based on these theories we assumed that chitosan and caffeine can perform this kind of interaction to associate with eachother.
3. 2 Materials and Methods

3.2.1. Materials

Chitosan with 75% deacetylation and a molecular weight $M_w < 150,000$ Da was obtained from Novamatrix, Norway (Protasan UPCL 113). Centrisart with a molecular weight cut off (MWCO) of 20,000 Da, Vivaspin 20 which a MWCO of 300,000 and 100,000 Da were obtained from Sartorius, Göttingen, Germany. Caffeine anhydrous was obtained from Sigma Aldrich, St Louis, USA. All other solvents and chemicals were commercially available, from the highest grade and used as obtained.

3.2.2. Equipment

UV-Vis Spectrophotometer, Lambda 35 and FTIR Spectrophotometer, Spectrum 400 series, PerkinElmer LAS Rodgau, Germany were used. Differential Scanning Calorimetry (DSC) was performed by using DSC-Q100 from TA Instrument, Germany. Sample holder for DSC from Hermetic Lid, Germany, Sonicator, Bandelin from Sonorex, Germany, Vortex Genie from Scientific Industries, Germany, Centrifuge, Rotina 420R from Hettich Zentrifugen, Germany, and pH meter from Schott, Germany was used for the different investigations.

3.2.3. FTIR measurement

To perform Fourier transform infrared spectroscopy (FTIR) measurements, physical mixture of chitosan and caffeine powder was used. For the comparison, the samples was also prepared from solution by dissolving Protasan UPCL 113 at pH 9 to obtain 0.3% chitosan solution with 10 mg of caffeine. While stirring, the interaction could take place for 30 minutes at room temperature followed by lyophilization. A total of 3 mg of this mixture was characterized by FTIR Spectrophotometry. Pure chitosan and caffeine anhydrate were also used as references.

3.2.4. DSC measurements

DSC measurements were taken under nitrogen flow of 50 -100 mL.min$^{-1}$ using a sample mass of 4 mg and heating rates of 10°C min$^{-1}$. The samples were placed into covered aluminum holders with a central pinhole. An empty sample holder was used as reference and the runs were performed by heating the samples from 25 up
to 400°C. The samples were the physical mixture of chitosan and caffeine, chitosan (Protasan UPCL 113) and caffeine were used for reference.

3.2.5 Determination of chitosan and caffeine by UV spectroscopy

Stock solution of chitosan chloride was obtained by dissolving 5 mg Protasan in 2 mL demineralized water. After that, into the chitosan solution, hydrochloride acid 0.1 M was added to adjust the concentration of chitosan to 0.25 mg/mL. From this solution, the determination of $\lambda_{\text{max}}$ was performed by UV spectrophotometer. The measurement was performed in quartz cuvettes between 200 and 300 nm using an absorption peak of chitosan [137]. For calibration, serial concentrations of chitosan in hydrochloride acid 0.1 M was used to obtain the concentrations of 0, 0.006, 0.125, 0.250, 0.350, 0.500, 0.650 and 0.750 mg/mL. For the solubilisation study, the permeation of chitosan through different filters was tested (Vivaspin 20 with MWCO 300,000 and 100,000 Da and Centrisart with MWCO 20,000 Da) and the concentration of chitosan passed through the membrane was calculated. For the preparation of caffeine solution, 5 mg caffeine was dissolve in demineralized water as stock solution. For calibration concentrations of caffeine of 0.0015, 0.003, 0.004, 0.006, 0.008, 0.012 and 0.024 mg/mL were used.

3.2.6 Solubilization study of caffeine in the presence of chitosan

First of all, caffeine in high amount was added to an aqueous phase at 25°C to determine the saturation concentration ($c_s$) keeping the temperature constant. For solubilization studies, the amount of caffeine was always kept higher than $c_s$.

![Figure 11: Scheme of the setup which was used for the determination of $c_s$ of caffeine using Centrisart 20 kDa to measure the solubility of caffeine.](image)
Determination of the dissolved amount of caffeine was conducted by adding different amounts of chitosan to the inner chamber of the Centrisart (Fig.11). A total of 60 mg of caffeine were suspended in 1.5 mL of demineralized water. The sample was transferred to the outer tube of the Centrisart. Then 0.5 mL of chitosan with several concentrations 0.075, 0.150 and 0.300 mg/mL were added separately to the inner tube with membrane MWCO 20,000 Da as shown in Fig.11 and followed by centrifugation with 1000 x g for 90 minutes. Hereafter, the amount of caffeine in the supernatant was determined by UV spectrophotometer.

3.3. Results and Discussion

3.3.1. Physical interaction between chitosan and caffeine

Protasan UPCL 113 used in this experiment, is commercially available, has an average degree of deacetylation of more than 75% and its molecular weight distribution ranges from 10,000 to 400,000 Da. This type of chitosan chloride is soluble in water also in pH range 5 to 9. At pH 11 Protasan precipitates. In the pH range where Protasan is soluble its amine (-NH$_2$) functional group can possibly perform an interaction with the carbonyl group (C=O) present in caffeine.

The determination of an interaction between chitosan and caffeine is the first indication which is a necessary for the loading of caffeine into chitosan-PLGA NPs. Therefore, the interaction between chitosan and caffeine was addressed looking at IR spectra and the glass transition temperature.

First of all, the samples were prepared and the interaction patterns were analyzed by using a procedure to determine caffeine [138]. As shown in Fig.12, the FTIR spectra display the typical peaks from the functional groups. The stretching bond of carbonyl is typically found in the range between 1447-1705 cm$^{-1}$ [139]. For caffeine, the stretching bond of carbonyl was found at 1646 cm$^{-1}$ right in the range as expected. The stretching bond of methylamine is typically found in the range between 992-1260 cm$^{-1}$ [140]. For chitosan, stretching bond of methylamine was found at 1020 cm$^{-1}$ also right in the range as expected. Both wavenumbers did not change their position for the mixture. The complex formation is supported by the change of intensity of the peaks in the spectrum allowing to conclude that the interaction involves hydrogen bonds between the amine groups of chitosan and the carbonyl groups of caffeine. Furthermore, it can be concluded, that the interaction does not
III. Interaction of Chitosan and Caffeine

involved formation of covalent bonds as there was no shift in the wavenumber (cm\(^{-1}\)) observed (Fig.12). Possible covalent bonds would occur between amine and carboxyl groups (HO-C=O) in the presence of high temperature. As caffeine only has a carbonyl group and the interaction took place at room temperature (~23°C) the formation of a covalent bond was not likely. The wavenumber above 2750 cm\(^{-1}\) represents the stretching of –OH and C-C of chitosan. Blue lines are the complex of chitosan and caffeine and the stretching –OH and C-C were revealed as shown in Fig.12.

In Fig.13 the DSC measurements are displayed. It can be seen that water evaporates at the peak below 100°C in the chitosan spectrum (grey line). Typically this water is associated with hydrophilic groups in the amorphous chitosan. Caffeine as anhydrous base did not show that peak (black line) as expected. Endothermic phase transition from solid to liquid took place below 250°C for chitosan and caffeine. The disappearance of the endothermic peak of caffeine at 310°C indicated that caffeine dissolved in the molten chitosan which is an indication that chitosan and caffeine form a complex.
III. Interaction of Chitosan and Caffeine

3.3.2 Solubilization study of caffeine by using chitosan

This step was performed to support the information that was already obtained regarding the physical interaction that was explained above. At first, the change of dispersion turbidity containing saturated caffeine was performed. Fig.14 shows that (Fig.14A), the saturated solution containing insoluble caffeine results in a turbid solution. Afterward, chitosan was added to this solution of caffeine resulting in clear solution (Fig.14B).

Figure 13. DSC measurements of pure chitosan, caffeine and both together to identify a possible physical interaction between caffeine and chitosan

Figure 14. Saturated caffeine become clear solution because of solubilization by adding chitosan.
A. Saturated solution containing caffeine
B. After adding chitosan
This reduction in turbidity is caused by additional dissolved, suspended caffeine by the presence of chitosan. Since the free caffeine interacts with chitosan, forming complexes, the non-dissolved, solid caffeine can dissolve to reach the saturation concentration.

Besides this first indication regarding turbidity, the capability of chitosan to solubilize caffeine was investigated. To test the solubilization capability of chitosan for caffeine, a filter setup was used. In this setup caffeine and chitosan were in two different compartments separated by a membrane. For the compartments Vivaspin system or Centrisart system was tested. Vivaspin has one tube and in that tube located the membrane with certain MWCO (300,000 and 100,000 Da). Protasan UPCL 113 has molecular weight <150,000 Da. The amount of chitosan passed through the membrane can influence the amount of caffeine which was solubilized in this experiment. Therefore, before going to the solubilization study, the determination of chitosan passing the membrane is necessary. Chitosan was measured using the absorption maximum at 207 nm in acidic solution of pH 1 (0.1 M HCl) [137]. At high wavelength close to 300 nm there was no absorption as shown in Fig.15. Therefore, the peak at 207 nm works well for the determination and quantification of chitosan as can be seen from the Fig.15.

![Figure 15. Absorption maximum of chitosan between 200 and 300 nm and the respective calibration curve of chitosan based on this absorption.](image-url)

Based on result in Fig.16, Vivaspin is not suitable for solubilization study because chitosan can pass through the membrane. On the other hand, Centrisart presents small MWCO to hold chitosan (Fig.16). With centrisart (20,000 Da) system, chitosan has not passed through the membrane.
III. Interaction of Chitosan and Caffeine

Figure 16. Amount of chitosan that could pass through the filter of Vivaspin and Centrisart with different MWCO.

Centrisart (20,000 Da) was investigated furthermore to perform solubilization study (hence allowed to determine the caffeine concentration with interference from the polymer). Subsequently, determination of chitosan and caffeine can be determined by using UV spectrophotometry.

In Fig. 17 we can see the peak of caffeine which also could be determined by using UV spectrophotometry. Caffeine has the maximum absorption at 272 nm in demineralized water. Based on the two spectra of chitosan and caffeine, it was concluded that chitosan did not interfere with the caffeine determination because chitosan does not absorb at 272 nm (Fig. 15).

![Graph showing concentration of chitosan and caffeine](image)

Figure 17. Local absorption maximum of caffeine at $\lambda = 272$ nm and the calibration curve of caffeine

To determine the amount of caffeine in solubilization study by using UV spectrometry, first of all the calibration curve of caffeine was made by serial
III. Interaction of Chitosan and Caffeine

concentration of caffeine as shown in Fig.17. The linear curve was achieved with $R^2 = 0.9989$. Based on this calibration curve, the amount of caffeine which was solubilized by chitosan could be calculated.

First of all the saturation concentration was determined as reference point for caffeine solubility at 25°C. In Fig.18 $c_s$ is shown as dashed line at a concentration of 26.2 mg/mL which is in agreement with literature data [141]. The concentrations of chitosan in contact with the saturated caffeine solution were then varied from 0.075 mg/mL to 0.30 mg/mL. Subsequently, using several concentration of chitosan ranging from low (0.075 mg/mL) to high (0.3 mg/mL), the solubility of caffeine was influenced by the concentration of chitosan. This trend was shown in Fig.18 that indicating roughly a linear concentration between chitosan concentration and additional dissolved caffeine amount ($R^2 = 0.9413$). Hence, chitosan clearly has the capability to solubilize caffeine.

![Graph showing the solubilization of saturated caffeine by adding chitosan.](image)

**Fig. 18: Solubilization of saturated caffeine by adding chitosan.**
Dashed line represents the saturation concentration of caffeine ($c_s$)

3.4 Conclusions

Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) measurements and a solubilization study could demonstrate an interaction between caffeine and chitosan. The elucidation of the interaction between caffeine and chitosan which was already investigated by FTIR measurement indicated that it was a complex formation and there was no peak shift of the carbonyl
group at 1646 cm\(^{-1}\) and the methyl amine group of chitosan at 1020 cm\(^{-1}\). The formation of such a complex was supported by DSC measurements where the glass transition temperature revealed that molten caffeine is soluble in molten chitosan at temperatures above 200°C. Furthermore, the changes observed in turbidity of the saturated solution of caffeine becoming clear when adding chitosan was another clear hint for this interaction taking place between caffeine and chitosan. UV measurements further allowed quantifying the effect of chitosan on the solubility of caffeine. Initially, without chitosan, the solubility of caffeine was 26.2 mg/mL in water at room temperature. By adding chitosan up to 0.3 mg/mL the concentration of caffeine solubilized was found to be 30.07 mg/mL. So, this amount of chitosan (0.3 mg/mL) could interact with caffeine and increase the saturation concentration by ~4 mg/mL.
IV. Nanoparticulate Formulation Intended for Hair Follicle Targeting

4. 1. Introduction

Investigation of the use of drug carrier systems to treat hair loss is still in early stage. In Human life, hair loss is a condition which occurs more frequent along with time and increasing activities of human leading to stress conditions during daily work. When a person experiences stress without realizing, so the stress condition lead to a weakened metabolic system. Another reason for hair loss is because of the natural aging process enhanced by hair care. After a hair is lost, usually the new hair can grow again; if not baldness will occur [142,143]. Shampoo containing caffeine, as a non-particulate formulation, has been available in commercial trade to stimulate hair growth for quite some time [31,128,144]. In general, hair follicles were believed to be a minor pathway for transdermal drug delivery into human skin [62,83,87,145] due to the small hair follicle density of an average of 0.1% [38]. Based on recent publications, it is known that particulate formulations show better penetration into hair follicles than formulations without particle [62,63]. Particulate formulations, micro and nanoparticles (NPs), which have been evaluated in several experiments about transfollicular penetration indicated that particles with an optimal size of 320 nm can penetrate deeply into the hair follicles [63]. However, for targeting of hair follicles, particles size from 500 to 600 nm are believed to be well suited [86]. Beside the possibility to penetrate into the hair follicles, the uptake into cells, especially Langerhans cells, was according to literature observed for particles of around 40 nm [146]. So depending on the specific need and target within the follicle different-sized particles could be utilized.

To achieve a good drug carrier system the applied materials (polymers) have a large impact. The utilization of natural and synthetic polymers is foremost to increase the capability of carrier systems for drug delivery. Until now, some natural polymers which have been successfully purified and synthetic polymers have been produced to serve the formulation of NPs [107,147,148]. There are several requirements for these polymers regarding regulative issues such as the biodegradability, biocompatibility and benign character (toxicity) as well as technological aspects such as the interaction between polymer and active pharmaceutical ingredient (API) influencing the release profile from the NPs. Based on these criteria, the polymers which were most extensively applied for creating nanoparticulate delivery systems over one
decade are poly(lactic-co-glycolic acid) (PLGA) and chitosan. NPs based on PLGA were usually prepared by using emulsion solvent diffusion techniques which can be considered the standard methodology. Nevertheless, PLGA NPs could also be prepared by nanoprecipitation, emulsion coalescence and spray drying depending on the specific goals of the formulation process [132,148-151].

It is also known that by using PLGA, a good encapsulation has been achieved for hydrophobic APIs. In contrast, the encapsulation of hydrophilic drugs is not an easy task and influenced by several factors such as kind of the polymer, the organic solvents and correctly chosen stabilizers [108,115,152-154]. Nevertheless, some studies reported, that by optimizing these factors, hydrophilic APIs could be loaded into PLGA NPs by nanoprecipitation [102,103]. A combination of PLGA and chitosan was chosen to vary the particle properties [110,111]. For delivery purposes, the presence of amine functional groups of chitosan allowed to make use of the electrostatic effect, of hydrogen bonds, and Van der Waals forces to interact with the carbonyl functional group of the bioactive compound [155,156].

In this study, preparation of chitosan-PLGA NPs was performed by using emulsion solvent diffusion method. Two types of homogenizer -ultrasonifier and ultraturrax- were evaluated as well as two types of chitosan: Protasan UPCL 113 and Kitozyme. In addition, PLGA NPs without chitosan were also prepared as a control. Based on the literature reports that the double emulsion method for preparation of NPs could load hydrophilic drugs, this method was also used for comparison. Pure chitosan NPs without PLGA were prepared by using the crosslinker tripolyphosphate (TPP). NPs were then characterized regarding size, polydispersity index, zeta potential and morphology. The appropriate formula in terms of size distribution and morphology was selected to be loaded with caffeine. Subsequently, NPs were purified and % encapsulation efficiency as indirect EE of caffeine from the supernatant was calculated. In terms of particles, the direct EE and loading of caffeine was determined after NPs were dissolved. And finally, the release profile of caffeine from the NPs was monitored.
4. 2. Materials and Methods

4.2.1. Materials

PLGA 50:50 (Resomer RG 503) with a molecular weight $M_w$ of 24,000 - 38,000 Da was obtained from Evonik Industries (Darmstadt, Germany). Chitosan chloride with a degree of 75% deacetylation (Protasan UPCL 113) with a molecular weight < 150,000 Da was obtained from Novamatrix (Norway), chitosan base (Kiomedeine®) with a degree of 75% deacetylation was obtained from Kitozyme (Herstal, Belgium). Polyvinyl alcohol (PVA) (Mowiol 4-28) was obtained from Kuraray specialties (Frankfurt, Germany), Pluronic F68 was obtained from BASF (Ludwigshafen, Germany), Tripolyphosphate (TPP) was bought from Merck, Darmstadt, Germany, cellulose membranes with a MWCO 12,000 – 14,000 Da were bought from Medicell International LTD (London, UK) and caffeine anhydrous came from Sigma Aldrich (St Louis, USA). For scanning force microscopy muskovite mica was obtained from Plano Planet GmbH, Wetzlar, Germany. Silica wafers were obtained from Wacker Chemie, Germany. NSC 16/50 non-contact silicon cantilevers, MikroMasch, Cambridge, UK. All other solvents and chemicals were commercially available and of highest grade.

4.2.2 Equipment

Zetasizer Nano ZS from Malvern Instruments, Worcestershire, UK was used to determine the size and the zeta potential of the particles. The morphology of NPs was determined by Atomic Force Microscopy (AFM) using a Nanoscope IV Controller from Digital Instruments, Bruker Corporation, Billerica, USA and scanning electron microscopy (SEM) using a EVO HD from Carl Zeiss, Jena, Germany.

4.2.3. Preparation of nanoparticles

The common method to form NPs loading hydrophilic drug is based on a double emulsion[100]. It was formed by using 2.5 mL PVA 2.5 mg/mL containing 6.25 mg/mL caffeine which was dropped into 40 mg/mL PLGA in 2.5 mL ethyl acetate. The mixture was then placed in an ice bath under stirring for 1 hour at 750 RPM. To produce a nanoemulsion, ultraturrax at 13,200 RPM was applied for 10 minutes. The second emulsification step was formed by using Pluronic F68 as
stabilizer with concentrations of 15 and 20 mg/mL (2.5 mL for each). The resulting emulsion was stirred over night at 500 RPM to evaporate the organic solvent.

Emulsion solvent diffusion method was used to prepare the chitosan-PLGA NPs as described before [110,111]. In brief, the emulsion was prepared by using PLGA 40 mg/mL in 2.5 mL ethyl acetate as organic phase. The aqueous phase consisted of 2.5 mL of 25 mg/mL PVA solution, chitosan 0.3 mg/mL, and 6.25 mg/mL caffeine. The aqueous phase was dropped into ethyl acetate as organic phase. The mixture was placed in an ice bath under stirring for 1 hour at 750 RPM. To produce a nanoemulsion, both ultrasonifier at 10% amplitude (A) with 500 Joule for 1 minute and ultraturrax at 13,200 RPM for 10 minutes in an ice bath were applied. The resulting emulsion was stirred over night at 500 RPM to evaporate the organic solvent. For size comparison, blank PLGA NPs and chitosan-PLGA NPs were prepared without caffeine using the same protocol as for the single-step emulsion solvent diffusion. Besides Protasan, another type of chitosan - Kiomedine originating from mushrooms and produced by Kitozyme was also used for comparison. Pure chitosan NPs without PLGA core were also prepared by using ionic gelation method based on the crosslinker tripolyphosphate (TPP). Chitosan (Protasan) was dissolved in demineralized water to make a stock solution of chitosan (0.625 mg/mL) and TPP was dissolved also in demineralized as stock solution of TPP (0.125 mg/mL). Chitosan NPs were formed by adding 2 mL TPP 0.125 mg/mL into 2 mL chitosan 0.625 mg/mL. The resulting mixture was stirred for 4 hours.

4.2.4. Purification of nanoparticles and %EE of caffeine

5 mL suspension of NPs was placed in Vivaspin-20 (Sartorius, Göttingen, Germany). The suspensions were purified by using a centrifuge at 14,000 x g at \( T = 8^\circ \text{C} \) for 30 minutes. The supernatant were collected to determine the encapsulation efficiency (%EE) of caffeine by using HPLC-analysis. The mobile phase of HPLC was a phosphate buffer pH 2.6 and acetonitrile (90:10) with a flow rate of 1.2 mL/min. The HPLC column was a reverse phase column C18 with controlled temperature at 25°C.

The amount of NPs was obtained from lyophilized samples. After that, this sample allows to quantify the loading of drug in the formulation. The loading can be calculated based on the amount of drug in NPs and the weight of the samples used.
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for investigation. For lyophilization, sample was pre-frozen at $T = -80^\circ C$ and then the drying process was continued by a conventional Alpha 2-4, freeze-dryer (Christ, Osterode, Germany).

4.2.5. Size, PDI and zeta potential and morphology study of the nanoparticles

Determination of size, PDI and zeta ($\zeta$) potential were the common approaches to determine the properties of the NPs. After evaporation of the organic solvent, the NP suspensions were characterized regarding size, $\zeta$-potential, and polydispersity index (PDI) using dynamic light scattering (DLS) from Malvern Instruments, Worcestershire, UK. NPs were purified by Vivaspin20 and the filtered NPs were washed with 0.2% trehalose which also acted for the pellet redispersant. The redispersed NPs were analyzed regarding their morphology. The morphology of NPs was visualized by AFM and SEM measurements. Before the measurements, NPs were diluted in demineralized water using vortex and dried overnight at room temperature. AFM measurements were done with a Bioscope with a Nanoscope IV controller (Veeco Instruments, Bruker, Germany). Samples were prepared by dropping 20 $\mu$L of diluted sample on freshly cleaved mica. The NPs were investigated after drying under ambient condition with tapping mode using a tip with cantilever of $k = 40$ N/m and a resonance frequency of $\approx 250$ kHz. For SEM measurements, the samples were dried under ambient conditions and coated with gold using a Quorum Q 150 ES sputter coater at 40 mA for 50 s in order to render the sample conductive, improving the image quality (thickness of the gold coating was less than 20 nm). For visualization a ZEISS EVO HD 15 was used in high vacuum mode, applying an acceleration voltage of 5 kV.

4.2.6. Dissolving nanoparticles and determination of loading

Several organic solvents such as ethyl acetate, dichloromethane, dimethylformamide (DMF), tetrahydrofuran (THF) and dimethylsulfoxide (DMSO) were tested to dissolve chitosan-PLGA NP. As a control, PLGA NPs were used in this experiment. PLGA dissolves in DMSO and ethyl acetate and chitosan is only soluble in acidic solution. DMSO is soluble in water but ethyl acetate is only partially soluble in water. The NPs were placed into 0.1% acetic acid, followed by DMSO to obtain a one-phase system. This approach required heat treatment at $T = 60^\circ C$ for 9
hours and 15 minutes sonication successively. Afterwards, the amount of caffeine loaded was determined (direct method).

4.2.7. In vitro release study

For the release study, Franz Diffusion Cells were set up. For the study the same volume of acceptor and donor compartment was used. The drug can diffuse effectively into the acceptor compartment and the speed of stirrer bar also the sink condition of each cell were important factors for release study using Franz Diffusion Cell. Performing release study followed the procedure described before [157]. The donor compartment was filled with 40 mg lyophilized NPs containing caffeine suspended in 0.5 mL demineralized water. A Medicell membrane with MWCO 14,000 Da was placed in between donor and acceptor compartment. The acceptor compartment was filled with 12.5 mL of phosphate buffered saline (PBS) pH 7.4. The caffeine released to the acceptor compartment at room temperature was sampled using a long syringe; a Pasteur pipette was used to refill the acceptor compartment through the sampling port. The amount of released caffeine was determined by HPLC analysis. In this determination, mobile phase phosphate buffer pH 2.6; acetonitrile = 90:10 was used, the retention time was 5.1 min, at $\lambda_{\text{max}}$ was 262 nm. The shift of $\lambda_{\text{max}}$ from 272 to 262 nm due to the influence of acetonitrile and phosphate buffer in mobile phase of HPLC. Flow rate was 1.2 mL/min and injection volume 20 µL. For comparison, the passive diffusion of pure caffeine and from a mixture of caffeine-chitosan was also determined.

4.3 Results and Discussion

4.3.1 Size, PDI, and zeta potential of the prepared NPs

After we established the binding capacity of chitosan to caffeine, we developed a formulation with NPs loading caffeine using biodegradable and biocompatible polymers (chitosan and PLGA) by using the established solvent diffusion evaporation method (single emulsion).

The common type of drug to load PLGA NPs is hydrophobic. Nevertheless, hydrophilic drugs can also be loaded into PLGA NPs using double emulsion[102,
Therefore double emulsion was also used to encapsulate caffeine as reference method.

Both methods -single and double emulsion- have used PLGA as polymer due to its well-known properties. Resomer RG 503 is a PLGA which has 50% glycolic and 50% of lactic acid groups and a molecular weight $M_w$ of 24,000 to 38,000 Da. Based on literature, PLGA in combination with chitosan is able to form PLGA NPs (core) coated with chitosan. This system has been described to be a good drug carrier system [110, 111, 115, 158]. In addition, chitosan-PLGA NPs prepared using the standard method (single emulsion) were described showing better PDI than other approaches. Besides the formulation of hybrid particles based on PLGA and chitosan, chitosan NP could also be formed by combining it with alginate, gelatine and crosslinker molecules. Furthermore, pure chitosan particles were prepared using the crosslinker tripolyphosphate (TPP) which was chosen due to its good safety profile.

Figure 19. Size and charge of PLGA, chitosan-PLGA, and TPP-chitosan particles. The dashed line represents a guide to the eye to show the reference size from pure PLGA NP.

First of all, double emulsion as common method to load polymeric particles with hydrophilic drugs was used to prepare PLGA NPs loading caffeine. This preparation method was then compared to a single emulsion preparation containing

![Figure 19](image-url)
forming chitosan-PLGA NPs using the specific interaction between chitosan and caffeine for loading. As shown in Fig. 19 size and ζ-potential of NPs prepared by the double emulsion methods with two concentrations of the stabilizer Pluronic F68 are depicted. The two double emulsion formulations resulted in particles smaller than 300 nm and with a PDI of 0.16 indicating a relatively broad size distribution of the carriers. However, single emulsion method for chitosan-coated PLGA NPs showed smaller sizes (d ~ 250 nm) and a very good PDI of ~0.06. As control, blank PLGA NP were prepared yielding the smallest and most well distributed particles with sizes of 208 nm and a PDI of 0.02. Particles prepared by the ionic gelation method (chit-TPP), using tripolyphosphate (TPP) showed sizes of more than 300 nm and the worst size distribution (PDI = 0.21) (Fig. 19). Looking a bit closer at the size distribution (Fig. 20) the chit-TPP NPs had the largest range with a full width at half maximum (FWHM) of 205.49 nm. The second largest FWHM was found for PLGA NPs prepared by double emulsion with particles showing a FWHM of 166.38 nm, followed by chit-PLGA NPs with FWHM = 155.25 nm and the control PLGA NPs with the smallest distribution (FWHM = 123.75 nm).

![Size and distribution of chit-PLGA, PLGA, chit-TPP and PLGA double emulsion](image)

Figure 20. Size and distribution of chit-PLGA, PLGA, chit-TPP and PLGA double emulsion

The zeta potential of the different preparations followed the expected trends displaying negative ζ-potentials for the preparations without chitosan and positive ζ-potentials for the chitosan containing formulation because of the amine groups of chitosan (Fig. 19).
IV. Nanoparticulate Formulation Intended for Hair Follicle Targeting

Based on the results of the characterization of NPs size, PDI and ζ-potential, especially chitosan-TPP NPs did not show good properties with a too broad distribution (PDI = 0.21). Therefore, these particles were not further investigated. The preparation of PLGA NP and the chitosan NP was further evaluated with respect to the dispersion method and the type of chitosan used.

<table>
<thead>
<tr>
<th>Table 1. Ultraturrax and Ultrasonifier as Homogenizer</th>
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<tbody>
<tr>
<td>Samples</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>PLGA NP</td>
</tr>
<tr>
<td>Chit-PLGA NP</td>
</tr>
</tbody>
</table>

The effect of homogenizing method on the properties of NPs is shown in Tab.1. There was no difference with respect to size and PDI found. However, using the ultraturrax the 10 minutes preparation time caused an increase in temperature to 20°C with ice bath and even to 42°C without ice bath. On the other hand, ultrasonifier used for 1 minute only showed an increase in temperature to 11°C with ice bath (without ice bath was 18°C) resulting also in NPs with very good particulate characteristics. Since this method was less time consuming while providing a reduced temperature effect and both methods gave comparable size distributions the method based on the ultrasonifier was chosen for further experiments.

<table>
<thead>
<tr>
<th>Table 2. Impact of the kind of chitosan (Protasan and Kitozyme) on the NP properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Protasan</td>
</tr>
<tr>
<td>Kitozyme</td>
</tr>
</tbody>
</table>

As there is more than one kind of chitosan - Protasan and Kitozyme- the impact of the two types of chitosan was studied. Protasan was able to produce NPs with better PDI than those prepared with Kitozyme (Tab.2). Overall the differences were small. However, one important disadvantage of Kitozyme is its solubility profile restricting the preparation. The preparation of Kitozyme solution was more time consuming because to dissolve this chitosan in acidic pH needs sonication for 2
minutes followed by stirring for 15 minutes. In contrast, Protasan dissolved immediately in demineralized water. To keep the temperature change of the process low, an ice bath was needed. In addition, the solubility of caffeine is also reduced by reducing temperature.

Hence lower preparation temperatures seemed to be meaningful for preparation as caffeine solubility changes. Taken all these data into consideration, chitosan-PLGA NPs which were formed by single emulsion method using Protasan and an ultrasonifier to form the nanoemulsion were chosen for drug loading. All further investigations as determination of the encapsulation efficiency, the loading capacity and the release of caffeine were performed with this formulation.

4.3.2. Purification of nanoparticles, %EE of caffeine and morphology study

Supernatant after purification of chitosan-PLGA NPs loading caffeine was collected. The amount of caffeine in the supernatant was quantified by using HPLC-analysis. EE was calculated based on the following formula:

$$ EE = \frac{\text{total amount of caff} - \text{amount of caff in supernatant}}{\text{total amount of caff}} \times 100\% $$

caff is caffeine

EE of caffeine from chitosan-PLGA NPs was found to be (19 ± 1.1)%. In comparison the EE of caffeine in PLGA NPs prepared by the double emulsion method was only (14 ± 1.1)%. Obviously, the addition of chitosan forming chitosan NPs using single emulsion led to an increased EE compared to the double emulsion approach.

Based on the AFM and SEM images it could be demonstrated that the morphology of chitosan-PLGA and PLGA NPs was spherical with a smooth surface (Fig. 21). From Fig.21, analysis of the average size of NPs was conducted by using Section Analysis of the Nanoscope software. Average size of NPs from AFM image (Fig.21a) of chit-PLGA NPs was 215.31 nm and the PLGA NPs (Fig.21b) were 147.8 nm.

The fact that the chit-PLGA NPs were larger in size was expected due to previous results [110] and the increased viscosity of the aqueous phase due to the
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presence of chitosan. Analysis of the average sizes of NPs using SEM images, chit-PLGA NPs (Fig.21c) yielded an average of 164.7 nm and the blank PLGA NPs (Fig.21d) were found to be 135.5 nm by using ImageJ. All the NPs from SEM images were smaller than the hydrodynamic sizes determined by light scattering because of the drying and vacuum process. This also explains the differences between SEM measurements, performed in vacuum, and AFM measurements done under ambient conditions. In addition, for AFM measurements the unknown effect of the tip geometry is always present increasing slightly the object size. Nevertheless, the particle sizes are all in the same size range within the variations due to the techniques and support the obtained narrow size distribution.

Figure 21. AFM images of chit-PLGA NPs (A), PLGA NPs (B) and SEM images of chit-PLGA (C) and PLGA NPs (D).
4.3.3. Dissolving of chitosan-PLGA nanoparticles to determine the loading

To determine the amount of drug incorporated in the particles it is necessary to dissolve the particles, evenly disperse the drug and then separate the drug from the other components for analysis. Therefore, the dissolution step of the particles is essential to evaluate directly the amount of loaded drug.

After purification and lyophilization of chitosan-PLGA NPs the samples were obtained as powders. To suspend them, demineralized water was added followed by vortexing. By this step, we obtained the NPs well suspended as a stable suspension. These suspensions were used for the dissolving step. However, dissolving the NPs is not always an easy task. For instance, in literature is already described that even plain PLGA NPs do not always completely dissolve by using good organic solvents such as DMSO [159]. Having this in mind, it was not surprising that the hybrid PLGA NPs with chitosan and stabilizers [160] were more difficult to dissolve. Chitosan, representing the outer layer of the particles [111], is well soluble in acidic solution.

Therefore an approach based on an immersion in an acidic solution was used first. An acetic acid solution 0.1% at $T = 60^\circ\text{C}$ for 9 hours was applied, followed by 15 minutes sonication. Afterwards, DMSO was added to the system to obtain an one-phase system of drug and polymeric entities (particles were completely dissolved). The successful dissolution of the NPs was investigated by turbidity measurement using UV/Vis spectroscopy. Before dissolution, the particles dispersed in water clearly showed a scattering signal (upper curve in Fig.22). After acetic acid-DMSO
treatment, the signal strongly drops indicating completely dissolved chitosan-PLGA NPs because of reduced or no colloidal scatteres. Light scattering data of the dissolving NP showed clear solution (no measured absorption signal in the range from 360-800 nm). This result showed that the problem regarding dissolving chitosan-PLGA NPs could be solved using such a two-step dissolution process. After knowing the condition to dissolve chitosan-PLGA NPs, we could continue to determine the amount of successfully loaded caffeine into the NPs. The solution of dissolved NPs was centrifuged at 14,000 x g for 90 minutes to remove large objects and filtered with Chromafil filter 0.2 µm to avoid agglomerated material eventually present. Afterward, 100 µL of this solution was diluted with 900 µL mobile phase (buffer phosphate pH 2.6; acetonitrile = 90:10). Detection wavelength was 262 nm with retention time of 5.1 min and flow rate was 1.2 mL/min with injection volume of 20 µL. The amount of caffeine was determined and the %EE by this direct method was found to be (12.33 ± 1.7094)% based on the following formula:

\[
\%EE_{\text{direct}} = \frac{\text{amount of caffeine in NPs}}{\text{amount of caffeine added in the beginning}} \times 100\
\]

The loading of caffeine was 0.059, indicating that in 100 mg NPs 5.9 mg caffeine were contained. The loading was calculated based on the following formula:

\[
\text{Loading} = \frac{\text{amount of caffeine in NPs}}{\text{total weight of NPs}}
\]

Based on this result, the %EE and the loading is low as typical of hydrophilic drug. However this values show that chit-PLGA NPs can load caffeine. Due to more specific localization of NPs in hair follicles, the small amount of NPs is sufficient.

4.3.4. In vitro release study

Franz Diffusion Cells were prepared to perform release studies according to a method which was described previously [161]. For this approach, lyophilized NPs loading caffeine were suspended in demineralized water and the caffeine released to the acceptor compartment at room temperature was monitored [157]. The sampling
was performed by a long syringe and Pasteur pipettes were used to refill the acceptor compartment through the sampling port. The membrane with MWCO 14,000 Da was placed in between acceptor and donor compartment. The concentration and hence the amount of released caffeine was determined by using HPLC analysis.

The influence of chitosan-PLGA NP loaded caffeine led to a prolonged and slower release of caffeine from the particles as shown in Fig.23. Up to 24 hours, 40% caffeine was rapidly released (burst release) and the remaining amount of caffeine was continuously released until at 56 h a plateau was reached. The missing amount of caffeine should be still associated with chitosan. Based on literature the encapsulation of drug into NPs can influence the pattern of drug release [162]. Obviously, chitosan coating PLGA NPs could reduce the burst release of caffeine from the particles. This is because of the interactions between caffeine and chitosan as shown in chapter 3. Furthermore, according to recent reports chitosan was found after freeze drying changing its swelling behavior. Chitosan was observed to show a time dependent hydration and swelling. As consequence the release of caffeine incorporated in such a swollen chitosan layer would be prolonged compared to the non-entrapped caffeine. Pure caffeine, as expected, showed the fastest passive diffusion across the membrane up to 6 h and achieved 75% released amount. For the mixture of caffeine-chitosan, passive diffusion of caffeine was slower in the first 6 hours than the diffusion across the membrane for pure caffeine but faster than the caffeine diffusion from the chit-PLGA NPs. After 24 hours the maximum of permeated caffeine was achieved at ~ 70%. This was the same level as found for free caffeine and represents the equilibrium level. The slower permeation across the membrane from the chit-PLGA NP might be due to the particulate nature. In addition to the interaction between chitosan and caffeine the geometric restrictions of the chitosan layer at the particle interface together with the swelling will contribute and further slow down the release from the particles. This permeation profile across the membrane indicated that caffeine was interacting with chitosan and that the resulting complex (caffeine-chitosan or caffeine-chit-PLGA NP) slowed down release and hence the free diffusion of the drug.
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Figure 23. Release/diffusion profiles of caffeine across a membrane for chit-PLGA NP loading caffeine, pure caffeine and the physical mixture of caffeine and chitosan.

4.4 Conclusions

In this study, we propose a new biodegradable nanocarrier for loading the hydrophilic drug caffeine. The NPs which were prepared showed spherical shape with sizes of 208±1.104 nm for pure PLGA, 254±2.621 nm for chitosan-PLGA, and 351±12.051 nm for TPP-chitosan. The polydispersity index of the two NP preparations based on PLGA was better than that of the ionic gelated chitosan. Therefore, this preparation method was not further investigated. The zeta potential of the NP based on PLGA was found to be negative whereas the chitosan-containing NPs were, as expected, positive. To load the hydrophilic drug the double emulsion approach and the single emulsion set-up were used and compared. The encapsulation efficiency of caffeine to the chitosan-PLGA NPs was determined to be (19 ± 1.1)%. It was higher than that found for the double emulsion method without chitosan (only ~14%). Thus the interaction between caffeine and chitosan enabled a higher loading. Therefore, this system seems to be better suited for an application targeting hair follicles. The loading of caffeine into chitosan-PLGA NP could also prolong the release of caffeine compared to the passive diffusion of caffeine and mixture of caffeine-chitosan. This reduced release of the drug could further support a reservoir effect of the particles when accumulating in hair follicles.
5.1 Introduction

To bring drugs across the skin is one of the biggest challenges for a drug delivery system [163-165]. The optimization of such systems is difficult, because skin is well known as a good barrier regarding to the dense, poorly permeable corneocytes and overlapping alignment of the cornified cell layers of skin. Another difficulty is that not all drugs could overcome the epidermis by penetration. It is dependent on the partition and diffusion coefficient of the active pharmaceutical ingredient (API). APIs with high partition coefficient can pass through the stratum corneum (SC) and for APIs with low partition coefficient it is difficult to penetrate SC. Molecular weight ($M_W$) of the API plays also an important role for skin penetration. 500 Dalton (Da) is the molar mass often stated as exclusion limit for dermal penetration and permeation through the SC [38]. Against these difficulties, the alternative strategy is to collect the API in the hair follicle as an intriguing approach gaining more attention for targeting API because the hair follicles are known as an efficient storage and penetration pathway for topically applied substances [145]. Follicular penetration for targeting with NPs is a relatively new topic of research. Using this pathway, the NP loaded with API can enter the hair follicles and then release the API without need to disturb the skin barrier (non-invasive delivery) [87,92,145].

Visualization of sectioned skin could be performed by using confocal laser scanning microscopy (CLSM). However imaging entire hair shafts in skin using CLSM faces drawbacks due to the size and the tilted orientation of the hair shaft in the skin [166]. Nevertheless, an imaging of intact hair follicles would be necessary and helpful to identifying the presence and location of the NPs. Usually sectioned skin was used for CLSM but finding intact hair follicles is a time consuming task by this technique. On the other hand, photoacoustic microscopy allows visualizing such large scale structure non-invasively [167-169]. A necessity for sufficient quality of images of photoacoustic microscopy is the usage of a contrast agent such as magnetite (iron oxide) [170]. Iron oxide can absorb the near infra red light and emit ultrasound. This ultrasound waves can then be detected and converted to an image by using an ultrasound-transducer [171,172]. Recently, researchers have focused to
load iron oxide into NP made from biodegradable polymer such as dextran, gelatin and PLGA to obtain paramagnetic properties of the NPs. These properties are especially advantageous with respect to cleaning and collecting of NPs [169,173,174]. The polymer of choice in this study is the synthetic polymer poly(lactic-co-glycolic acid) (PLGA) which is a well-known biodegradable and biocompatible pharma polymer [96,175,176]. This property is a basic requirement for therapeutic usage of NPs [44,99,106]. Thus magnetite is intended to be incorporated into PLGA NPs drug carrier.

Another advantage for the application of NP as drug delivery system is the reduction of undesired side effects of the API [177-179]. A controlled size distribution is a central requirement for a pharmaceutical product [180-182]. Stronger restrictions are laid upon the selection of the methods that will be used for NP production, avoiding harmful substances during the production process. Additionally, the stability of NP should be considered for the delivery system [183,184].

For the preparation of NPs, the emulsion solvent diffusion and nanoprecipitation are known as common methods [100,102,184]. Emulsion solvent diffusion and nanoprecipitation methods were used to prepare NPs. The effect of magnetite concentration on the formulation was investigated, in terms of the physical properties of NPs. Hydrodynamic size, dispersity index and zeta potential were determined by dynamic light scattering (DLS). The morphology of NPs was determined by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The localization of magnetite in the PLGA NPs was determined by AFM and transmission electron microscopy (TEM). Furthermore, the magnetite-PLGA NPs were applied for imaging of the model hair shaft by using photoacoustic microscopy.

5.2. Materials and Methods

5.2.1. Materials

PLGA Resomer RG 503 (50:50) was obtained from Evonik Industries. Its molecular weight is 24,000 - 38,000 Da. Polyvinyl alcohol (PVA) Mowiol was obtained from Kuraray, Pluronic F68 was obtained from BASF, agarose and oleic acid-stabilized magnetite in toluene were purchased from Sigma Aldrich, aminohexanamine-stabilized magnetite in dimethyl formamide (DMF) was obtained from the Department of Physical Chemistry, Saarland University. For scanning force microscopy, as sample support muskovite mica was obtained from Plano Planet.
GmbH, Wetzlar, Germany. Silica wafer was obtained from Wacker Chemie, Germany. NSC 16/50 non-contact silicon cantilevers from MikroMasch, Cambridge. Syringes (Sterican-26G) with diameter of 0.45 mm were obtained from Braun Medical AG, Emmenbrücke, Germany. All other solvents and chemicals were from the highest grade and commercially available.

5.2.2. Equipment

Zetasizer Nano ZS from Malvern Instruments, Worcestershire, UK. Atomic Force Microscope (AFM) with a Nanoscope IV (Digital Instruments), Bruker Corporation, Billerica, USA. Scanning electron microscopy (SEM) using a SEM EVO HD 15 from Carl Zeiss and sputter Quorum Q 150 RES from Judges scientific, UK. Transmission Electron Microscopy (TEM) JEM-2010, JEOL GmbH, Eching, Germany. Confocal laser scanning microscope ZEISS LSM 510 META, Carl Zeiss, Jena, Germany. Optoacoustic signals of pulsed Nd-YAG laser system (H7000) was provided an optical microscope (Olympus IX 81), Tokyo, Japan.

5.2.3 Preparation of PLGA NPs loaded with aminohexanamine-stabilized magnetite

To prepare nanoparticles containing magnetite, nanoprecipitation method [185] was used. 25 mg/mL PLGA and 1 mg/mL of magnetite particles were dispersed in acetone under stirring for 30 minutes. Nanoprecipitated particles occurred when this organic phase was immersed to an aqueous phase contained Pluronic F68 as stabilizer. The immersion process was conducted by using a syringe. Then acetone was evaporated overnight at room temperature (~22°C).

To compare with nanoprecipitation another method for the preparation of NPs -the single emulsion solvent diffusion- was used [116,169]. In brief, 25 mg/mL PLGA was dissolved in ethyl acetate as organic phase. After that, 1 mg/mL aminohexanamine-stabilized magnetite in DMF was added to 6 mL of the organic phase under stirring followed by sonication for 30 minutes and vortex 2 minutes to achieve a homogenous suspension. To form an emulsion, the organic phase was dropped to the aqueous phase containing 2.5% PVA as stabilizing agent and stirred for 1 hour. By using an ultrasonifier with 500 J for 30 seconds, the emulsion was homogenized to form sizes in nm scale. Afterwards, dilution by adding 6 mL demineralized water allowed ethyl acetate to mix with the aqueous phase. After that, ethyl acetate was evaporated overnight at room temperature (~22°C). The
preparation was conducted by using several concentrations of magnetite: 0.5, 1, 2, 4 and 6 mg/mL.

To homogenize aminohexanamine-stabilizing magnetite into organic phase in presence of DMF, several steps were needed. Regarding this limitation, in a second approach, it was tried to remove and exchange DMF. Thus, aminohexanamine stabilizing-magnetite in DMF was diluted in isopropanol and then poured into ethyl acetate containing 25 mg/mL PLGA. The supernatant was separated from precipitated magnetite-PLGA particles. The precipitate was dispersed in 2.5 mL ethyl acetate. Once dispersed, the single emulsion method was conducted. Organic phase was added drop by drop by using peristaltic pump into the aqueous phase containing 2.5% PVA. To emulsify this mixture, ultrasonifier with 500 J for 30 seconds was applied, followed by dilution with 6 mL demineralized water. Evaporation of the excess organic solvent was conducted at room temperature (≈22°C) over night.

5.2.4 Preparation of PLGA NP loaded with oleic acid stabilized-magnetite

In the previous step, the influence of removing DMF was removed. In this step, for comparison, the preparation of NPs was performed with oleic acid-stabilized magnetite in toluene which is miscible with ethyl acetate. NPs were prepared by using 25 mg/mL PLGA in ethyl acetate with 0.5, 1 and 2 mg/mL of oleic acid-stabilized magnetite. The organic phase was mixed by stirring and then dropped into PVA 2.5% as aqueous phase by using peristaltic pump. Stirring was continued for 1 hour. To form nano-sized NPs, homogenization by using ultrasonifier with 500 J for 30 seconds was applied. After that, dilution was conducted by adding 6 mL demineralized water. Over night evaporation was applied at room temperature (≈22°C) to remove ethyl acetate.

5.2.5 Purification and characterization of nanoparticles

For purification, the suspension of NPs as much as 5 ml was placed in Vivaspin-20 with a MWCO of 300,000 Da from Sartorius. The suspensions were purified by using a centrifuge at 14,000 x g, T = 8°C, for 30 minutes. Filtered NPs were washed and redispersed with demineralized water. For determination of the physical properties of resuspended NPs, they were characterized regarding size, polydispersity index (PDI) and zeta potential by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Morphologic characterization
was performed by SEM an AFM. To achieve an adequate concentration of NPs for the microscopic measurement, suspension was diluted 1:100 in demineralized water. Then, for SEM measurement, 20 µL of the diluted sample was dropped on a silica wafer and dried under ambient conditions. The sample was then coated with a layer of ≤ 20 nm gold (sputter coater Quorum Q 150 ES at 40 mA for 50 s) in order to render the sample conductive and improve image quality. For visualization a ZEISS EVO HD 15 SEM was used in the high vacuum mode, applying an acceleration voltage of 5 kV.

To obtain 3 dimensional information on the sample (20 µL of the diluted sample was dried on the freshly cleaved mica) an atomic force microscope (Bioscope SPM) with a Nanoscope IV controller (Digital Instruments, Bruker) was used. The NPs were investigated under ambient conditions in tapping mode using a tip with cantilever of k = 40 N/m, resonance frequency of ~250 kHz. After that, the AFM images were analyzed with the AFM software (Nanoscope IV version 5.12R.3).

5.2.6 Determination of the magnetite core by using TEM, elemental analysis and AFM phase imaging

Determination of magnetite core incorporated into PLGA NPs was conducted by TEM, elemental analysis and AFM phase imaging. TEM sample was prepared by dropping the 20 µL of magnetite-PLGA NPs suspension on a copper grid. The sample then was dried under ambient conditions on the surface of the grid. The visualization was conducted by JOEL Transmission Electron Microscopy (model JEM-2010 instrument, JEOL GmbH, Germany). The size of the magnetite NPs was determined from the contrast (black spots) at the TEM image. Furthermore, elemental analysis allowed the identification of the materials which were incorporated into PLGA particles.

5.2.7 Preparation of model hair shaft

The idea was to create a void structure (mimicking the hair shaft) which was then filled with PLGA NPs loading magnetite. Therefore a syringe was immersed into 1.75 % agarose. After the agarose solidified, a void was obtained by withdrawal of the syringe. The model is composed of three layers (Fig.24). First was a based layer (as a foundation), second was a gel forming layer containing the void, and the third was a cover layer (to restrict diffusion of NPs during the CLSM and photoacoustic measurement). First of all, to form the based layer, 5 mL agarose was poured into
Petri dish. After that, to form the gel layer, 12 mL agarose was added above the based layer. In this layer a syringe containing the suspension of NP was immersed before agarose could become solid. The model hair shaft was obtained when the agarose became solid around the syringe. After that, the suspension of NP was injected slowly into the void while the syringe was removed from the agarose. Therefore the void was filled with the NPs suspension. The whole surface was then covered with a thin layer of agarose. As control, pure magnetite and blank PLGA NPs were used to perform photoacoustic imaging. Whereas for the CLSM imaging, the PLGA NPs labeled with fluoresceinamine were used.

![Figure 24. Sketch of the reparation of model hair shaft containing magnetite-PLGA NPs](image)

### 5.2.8 Imaging model hair shaft by using CLSM and Photoacoustic Microscopy

For CLSM measurement using a confocal laser scanning microscope ZEISS LSM 510 META, Carl Zeiss, Jena, Germany, agarose gel samples were sliced in cubic blocks and placed on the cover slip. The slice was placed in the microscope with the model hair shaft being parallel to the objective. This preparation was necessary to omit the limitations regarding the focal distance of the objective. The excitation was performed by using $\lambda = 488$ nm. The fluorescence signal was collected after a band-pass 522/35.

Optoacoustic imaging is a selectively new method that relies on a good absorption of light (in near infrared region). The sample is irradiated by short laser pulses in the sub-nanosecond range generating broadband acoustic signals as a result of the thermoelastic effect. These signals propagate through the sample and can be acquired with adequate ultrasound transducers. When compared with
conventional ultrasound imaging, optoacoustic techniques have the advantage of higher contrast since optoacoustic signal amplitudes are proportional to the local absorption coefficient $\mu_a$. Since the absorption is dependent on the concentration of absorbing NPs, this effect can be used in order to assess the suitability of different particle types (magnetite, magnetite-loaded polymer) as contrast agents.

For assessing the detectability of the different NPs types, an optoacoustic microscope has been used. Photoacoustic microscopy setup was developed by Fraunhofer Institute for Biomedical Technology (IBMT), ST Ingbert, Germany. Optoacoustic signals were generated by a pulsed Neodymium-doped Yttrium Aluminum Garnet (Nd-YAG) laser system (H7000). The data were amplified using a single channel hardware platform (AMI-US/OA, Kibero GmbH) to obtain the images[186]. For imaging of the prepared samples (model hair shaft loaded with different NPs), two different optoacoustic imaging systems were used. The device developed by Fraunhofer IBMT is based on an inverted optical microscope (Olympus IX 81, Tokyo, Japan). It can be used as conventional optic microscope, acoustic microscope or optoacoustic microscope. For photoacoustic microscopy, the measurement could directly be conducted from the Petri dish containing the gel and the model hair shafts.

For acquisition of optoacoustic signals, an acoustic detection unit has been mounted to the microscope. It is based on a 2D piezo-scanner that allows point-wise acquisition of signals from the investigated sample. Signals can be acquired using focused high frequency ultrasound transducers. For achieving the highest resolution, a 400 MHz ZnO transducer with a lateral resolution of 2.5 µm developed by Fraunhofer IBMT is used [170]. The scanner and the transducer are attached to a rotating column so that a fast exchange between the optoacoustic/ultrasound modality and the conventional optical imaging mode is guaranteed. This is especially helpful when optical microscopy was used to assist the position of targeting the sample.

5.3 Results and Discussion

5.3.1 Preparation and characterization of size, PDI and zeta potential

In general, the formation magnetite loaded NPs could be realized by single emulsion and nanoprecipitation [169,185]. Therefore, these methods have been
chosen to prepare the formulation of PLGA NPs loading magnetite for photoacoustic imaging and drug delivery [185,187].

Applying the two different preparation methods, particles with clearly different properties were obtained (Tab.3). By using nanoprecipitation, the resulting NPs showed a broad size distribution (PDI = 0.53 ± 0.0323) even though the mean diameter was around 100 nm. As expected, using PLGA, the particles have negative zeta potential (-8.01 ± 0.81) mV [169].

Table 3. Hydrodynamic mean size (nm), PDI and Zeta Potential (mV) of PLGA NPs loading Magnetite were prepared by nanoprecipitation and single emulsion method

<table>
<thead>
<tr>
<th>Methods</th>
<th>Mean Size (nm ± SD)</th>
<th>Polydispersity Index (PDI ± SD)</th>
<th>Zeta Potential (mV ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoprecipitation</td>
<td>115.33 ± 5.01</td>
<td>0.53 ± 0.032</td>
<td>-8.01 ± 0.81</td>
</tr>
<tr>
<td>Single Emulsion</td>
<td>254.38 ± 3.88</td>
<td>0.06 ± 0.027</td>
<td>-5.44 ± 0.42</td>
</tr>
</tbody>
</table>

Standard deviation (SD) of (n=3)

On the other hand by using the same concentration of magnetite, single emulsion resulted in NP with good properties in terms of size and PDI. The size with 250 nm of the particles was much larger than from the nanoprecipitation. The particles were mostly monodispersly distributed as can be seen (Tab.3) from the small PDI below 0.1 (PDI = 0.060). From this part, we chose the single emulsion method for further preparations. The influence of magnetite concentration was then evaluated for concentrations ranging from 0.5 – 6 mg/mL. Looking at the data (Tab.4), only the concentrations of magnetite lower than 2 mg/ml resulted in nanoparticles with small PDI values (monodisperse). High concentrations (4-6 mg/mL) resulted in polydisperse NPs (PDI>0.2). Based on this result, it was concluded that single emulsion method using magnetite up to 2 mg/mL is a well defined preparation which could be applied for the main work.

Table 4. Hydrodynamic mean size (nm), PDI and zeta potential (mV) of NPs loading magnetite prepared by single emulsion method.

<table>
<thead>
<tr>
<th>Concentration of magnetite</th>
<th>Mean Size (nm ± SD)</th>
<th>Polydispersity Index (PDI ± SD)</th>
<th>Zeta Potential (mV ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/mL</td>
<td>212.18 ± 2.16</td>
<td>0.08 ± 0.02</td>
<td>-5.91 ± 0.35</td>
</tr>
<tr>
<td>1 mg/mL</td>
<td>254.38 ± 3.88</td>
<td>0.06 ± 0.03</td>
<td>-5.44 ± 0.42</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>336.29 ± 6.21</td>
<td>0.12 ± 0.04</td>
<td>-5.34 ± 0.61</td>
</tr>
<tr>
<td>4 mg/mL</td>
<td>446.29 ± 8.28</td>
<td>0.24 ± 0.06</td>
<td>-4.06 ± 0.72</td>
</tr>
<tr>
<td>6 mg/mL</td>
<td>1174.3 ± 12.68</td>
<td>0.54 ± 0.08</td>
<td>-3.51 ± 0.75</td>
</tr>
</tbody>
</table>

Standard deviation (SD) of (n=3)
Solvent extraction method which removed the dimethyl formamide (DMF) of magnetite stabilized with aminohexanamine, could produce particles with good size and PDI. In the presence of DMF, to mix magnetite with ethyl acetate needs several steps such vortex and sonication. Therefore for the following preparation of NPs, DMF was removed. For comparison, oleic acid stabilized magnetite in toluene which is miscible in ethyl acetate was also utilized. Single emulsion method and concentrations of magnetite up to 2 mg/mL were applied (based on the previous result).

The monodisperse magnetite-PLGA NPs were obtained by using 0.5, 1.0 and 2.0 mg/mL (Fig.25) of two kind of magnetite (amino hexanamine-stabilized magnetite and oleic acid-stabilized magnetite). With a low concentration of magnetite (0.5 mg/mL) the mean size of particles was around 200 nm, the PDI values indicating a narrow distribution and the zeta potentials were slightly negative (for both kinds of magnetite used). Using 1 mg/mL magnetite could also result in monodisperse particles. These particles have mean size around 220 nm with narrow distributions (PDI 0.04) and they have also slightly negative zeta potentials (-5.616 mV). This method was obviously suitable for the preparation of PLGA NPs loading both oleic acid-stabilized magnetite in toluene (commercially available magnetite from Sigma Aldrich) and
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aminohexanamine-stabilized magnetite (from Department of Physical Chemistry, Saarland University). The physical properties of these magnetite-PLGA NPs are described in Fig.25. According to Fig.25, monodisperse NPs were obtained with magnetite of 1 mg/mL by using single emulsion method. Therefore, these NPs were utilized for microscopic characterization and hair shafts imaging.

5.3.2 Imaging NPs by SEM, AFM and TEM

Based on the result of the zetasizer measurements, all images were taken from NPs which were prepared by 1 mg/mL magnetite using single emulsion method. SEM and AFM measurements in order to know the morphology of the NPs which were diluted in demineralized water.

The SEM images (Fig.26) reveal that the NPs were spherical and the mean size was around 176 ± 6.92 nm calculated by freeware ImageJ (Version 1.46). The AFM image shown in Fig.27 allows to determine the mean size of NPs around 192 ± 17.74 nm by section analysis. Both sizes fit to the size range of particles applicable to hair follicles. It was also indicating that these sizes were smaller than the hydrodynamic size. This might be a result of the drying process during sample preparation for AFM and SEM measurements.

![Figure 26. SEM images of the magnetite-PLGA NP](image)

Both SEM and AFM images showed spherical particles well distributed with respect to their size. As depicted in Fig.27, NP was analyzed regarding their size by marking the bottom and top of the pancake-like structure by using section analysis. According to this image s was assumed that the distance between the two arrows indicated the high of collapsed particle containing a magnetite core.
The height was measured to be ~31 nm being a bit larger than the diameter of pure magnetite NP of ~20 nm (Fig.30). The differences in the value are due to the polymer surrounding the magnetite particles. It can also be seen that the collapsed particle is composed out of a thin ring (polymer) and a higher center. From this structure it can be concluded that the PLGA NPs contain a solid core, the magnetite particles.

5.3.3 Determination of the successful loading of magnetite in PLGA NPs

Even though we got some indication of the magnetite being incorporated in the PLGA particles, the key question is still to ensure if the magnetite was successfully entrapped. To answer the question, TEM, elemental analysis and AFM phase imaging were applied. TEM images in Fig.28 demonstrate that the grey spherical PLGA NPs (low contrast) contain black spots (high contrast) being most likely magnetite (Fig.28A).

The dense particle of magnetite demonstrated a low electron transmittance which corresponded to the black spot. In comparison, PLGA as organic polymer has high transmittance therefore the NP was seen as the grey particle. Three analyzed particles containing magnetite in Fig.28A have the size of approx. 155, 225 and 125 nm which were analyzed by ImageJ.
To identify the iron oxide, elemental analysis was conducted on one particle loading magnetite. The NP is a single particle and quite far from another NP therefore free space could be used as control and the information based on this data that iron peaks has come from PLGA NP loading magnetite. The strong iron signal from PLGA NP loading magnetite came from magnetite core (Fig. 28B) and the TEM grids did not have the spectra of iron. The copper and chrome signal were originating from the TEM grids (Fig. 28C).

For comparison with TEM, the phase images of AFM were taken and analyzed (Fig. 29). AFM phase imaging (Fig. 29B) refers to record the phase shift signal of tip oscillation during tapping measurement. The phase shift presents the delay in oscillation of the cantilever because of different material (adhesion and stiffness). The images indicate that PLGA NPs contain smaller objects. Together with the TEM images and the data of the elemental analysis that the observed black spots loaded in PLGA NPs are magnetite (Fig. 29A), these findings (TEM image and phase image of AFM), could be summed that NPs were formed by two kinds of different substances [188,189]. Based on the data of TEM and elemental analysis of iron
signal (Fig.28 A,B,C) also the AFM phase imaging (Fig.29B) it was concluded that magnetite was successfully loaded into the PLGA particles.

Figure 29. Determination of magnetite core on PLGA NP by TEM and AFM

Figure 30. TEM image of magnetite NPs

5.3.4 Imaging model hair shaft by using CLSM

Imaging of entire/intact hair shafts has been not done yet. The limitation is regarding to the size and orientation of the hair shaft. Before going to the complex system such hair shaft in an excised human skin, we tried to create a simple and appropriate model of a hair shaft in terms of the length and orientation. The model was created based on agarose (subtitle 5.2.7). In this work we also performed confocal imaging as control. This measurement was conducted on several model hair shafts which were filled by fluorescently labeled PLGA NPs applied as cubic sliced agarose gel blocks. For these experiments different orientations of the model hair shafts were used as illustrated in Fig.31. It is important in order to obtain the good images of the model hair shaft because of limitations regarding the focal distance of the objective.
In Fig.32 is shown the longitudinal position of model hair shaft. It is also clearly evidenced that visualizing the model from the top to bottom was not possible. This is because of the limitation of penetration depth of light and the restricted working distance of the microscope.

Inside of the model hair shaft, the NPs could not be seen clearly because the void was covered by one layer of agarose when the top of model was faced objective lens. The diameter of void is ~0.5 mm (Fig.32) in the range of diameter of human hair follicles 0.1 - 0.5 mm [190,191] . Only for the lateral orientation the model hair shaft could be imaged in parts. The composed images are shown in Fig.33. The distribution of NPs in the model was not homogeneous. Some of NPs were agglomerated as seen as large and bright fluorescent spots. However, it could not be fully compared to the hair shaft in terms of the orientation and length not being fully representative to the real situation. Furthermore, the imaging using several single CLSM images to compose the structure of interest is time consuming.
5.3.5 Imaging model hair shafts by using photoacoustic microscopy

To answer the difficulties of imaging model hair shaft we introduced a promising method to image model hair shafts: photoacoustic microscopy. The measurement followed the procedure described before (section 5.2.8).

Because of the high absorption of magnetite allowing to produce ultrasound when the laser light was applied, the samples should allow for successful imaging. The produced ultrasound was transferred as an image by using ultrasound transducer and the model hair shaft could be visualized.

Therefore, pure magnetite particles (Fig.34C) and PLGA NPs loading magnetite (Fig.34B) were filled in the model as control blank PLGA NP were used (Fig.34A). The photoacoustic images are shown in Fig.34. The measurement was quite fast and relatively easy because taking the image of the different model hair shafts from the top could be conducted simultaneously. Imaging reconstruction also allows displaying the side view of model hair shaft as showed in Fig.35.
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Figure 34. Top view of photoacoustic image of three model hair shafts containing particles. A) pure PLGA particles for control B) magnetite-PLGA NP and C) pure magnetite particles.

The tilted orientation is a common position with an angle around 45 to 60° for hair shafts [192,193]. The size and the orientation did not cause any problems in imaging. Based on Fig.35, the length of the model hair shaft is around 1.1 mm.

Due to the length distribution of human hair follicles in the range of 0.5-3 mm [194], we assumed that 1.1 mm is a valid size for our model being at least in the right range. Additionally, it was seen, that the covering layer made of agarose does not limit the imaging process in terms of the depth and the size of model hair shaft.

Figure 35. Photoacoustic images representing a side view of a model hair shaft containing magnetite-PLGA NP (red ellipse), the dashed line indicates the location of the covering layer.
5.4. Conclusions

In the present investigation, the formulation of PLGA NPs loading magnetite was obtained. The NPs were prepared by nanoprecipitation and single emulsion methods. By using single emulsion method in contrast to nanoprecipitation, spherical particles could be produced with a narrow distribution. By applying higher concentrations of magnetite (≥2 mg/mL) the NPs have shown broad unwanted distribution of particles. The successful incorporation of magnetite into the polymer NPs could be determined by using AFM and TEM images in combination elemental analysis. PLGA NPs loading magnetite were then applied to visualize model hair shafts embedded in agarose gel representing the hair follicle dimensions. The model hair shafts containing PLGA NP loading magnetite could be easily imaged by photoacoustic microscopy. Hence photoacoustic microscopy is an appropriate and promising technique to visualize intact hair follicles as large objects. This technique could be used to overcome the limitations of classical CLSM technique regarding the large scale and orientation of hair follicles for determining the localization of drug carrier systems.
VI. SUMMARY

Hair loss is a case which can be found in men and women. Usually, if hair loss occurs in an everyday manner, it will grow again by regeneration of new hair. When the amount of hair loss is more than the amount of hair growth, it will decrease the total amount of hairs. This condition could happen everyday and cause baldness. Feared by everyone, baldness is caused by continuous hair loss. Disruption of hair growth is influenced by internal and external factors of human body. Baldness is a rare case. Although rare, this is a worrying case for the patients. Nowadays, it has been attempted the way to prevent and treat hair loss by commercial products such as lotions, creams and shampoos. These commercial products containing caffeine in non particulate formulations are available on the market. Based on the recent research, it was known that the particulate formulation has better penetration into the hair follicles. A particulate formulation still needs to be developed, especially a nanoparticulate formulation which is promising product for hair follicles application. The investigations of this kind of formulation have been indicating the significant progress. Some of these investigations are aimed to develop pharmaceutical dosage forms because only a limited number of them employed nanoparticles (NPs). In the pharmaceutical field, NPs are produced with a uniform size. This NPs loading drug are intended to deliver and target the drug to the human body. Because NPs can reach the specific target, this should be able to diminish the side effect of drug.

Based on these informations, this study aimed to develop and characterize a particulate formulation by utilizing a biocompatible and biodegradable polymer such as chitosan and poly(lactic-co-glicolic acid) (PLGA) to form NPs loading caffeine. Interaction between chitosan and caffeine was determined by Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC) and by a solubilization study of caffeine. FTIR and DSC measurements indicated that the interaction between chitosan and caffeine is based on the formation of a complex. The solubilization study
showed the ability of chitosan to solve insoluble caffeine in a saturated dispersion and increase the saturation concentration. NPs loading caffeine were prepared by combining chitosan and PLGA to form chitosan-PLGA NPs (by single emulsion method) and solely PLGA NPs (by double emulsion method). Data of NPs characterization showed that NPs have a spherical shape. Especially, NPs made from chitosan-PLGA showed better properties than that of PLGA NPs in terms of particle size and size distribution. Loading of caffeine was increased by using chitosan. Caffeine release from NPs is slower than passive diffusion of solved caffeine which is not formulated in the form of particles.

The difficulty of delivering drug across the skin is prevalent. One of the strategies gaining more attention over the last years is the targeting of the hair follicles. Upon application the carrier system accumulates in the hair follicles and can release its load. The common/standard visualization technique is confocal laser scanning microscopy, but it has several problems originating from the physical size of the hair follicles and their orientation in skin. An intriguing alternative to fluorescence-based techniques is photoacoustic microscopy. Optoacoustic imaging is a novel method that based on the specific absorption of light in the near infrared region. The absorber such as iron oxide is irradiated by short laser pulses in the sub-nanosecond range generating broadband acoustic signals as a result of the thermoelastic effect. These signals propagating through the samples can be obtained with adequate ultrasound transducers. When compared with conventional ultrasound imaging, optoacoustic techniques have the advantage of higher contrast. Optoacoustic signal amplitudes are proportional to the local absorption coefficient $\mu_a$. Since the absorptions coefficient is dependent on the concentration of absorbing material, this effect can be utilized in order to assess the suitability of different material types (magnetite or magnetite-loaded polymer) as contrast agents.

As a possible label a magnetite core was introduced to the polymeric PLGA particles synthesized for drug delivery purposes. This combination of materials allows achieving magnetic properties as well as biocompatibility and biodegradability of the carrier. Furthermore, a good model system mimicking hair follicles would facilitate ex vivo investigations. Hence, preparation and characterization of magnetite PLGA NPs was in focus and the evaluation of a model hair shaft which could be imaged by
photoacoustic microscopy. The model hair shaft was prepared by using a syringe which was immersed into a 1.75% agarose gel (in a Petri dish). After solidification and retraction of the syringe, a void had been formed (serving as the model). This void was then filled with a suspension of NPs. The results showed that magnetite-PLGA NPs were successfully produced by utilizing 1 mg/mL magnetite and the model hair shaft containing magnetite-PLGA could consequently be imaged by using photoacoustic microscopy while confocal microscopy imaging was time consuming and complicated which could not image the large structure.
References


ABBREVIATIONS

AFM  = Atomic force microscopy
API  = Active pharmaceutical ingredient
AuNPs = Gold nanoparticles
cAMP = Cyclic adenosine monophosphate
Chit = Chitosan
CLSM = Confocal laser scanning microscopy
Da  = Dalton
DHT  = Dihydrotestosterone
DLS  = Dynamic light scattering
DMF  = Dimethyl formamide
DMSO = Dimethyl Sulfoxide
DSC  = Differential scanning calorimetry
EE  = Encapsulation efficiency
FDA  = Food and drug administration
FDC  = Franz diffusion cell
FTIR = Fourier transform infrared
FWHM = Full width half maximum
HF  = Hair follicles
HPLC = High performance liquid chromatography
MNPs = Magnetite nanoparticles
MRI  = Magnetic resonance imaging
MT  = Magnetite
MWCO= Molecular weight cut off
NPs  = Nanoparticles
PAM  = Photoacoustic Microscopy
PBS  = Phosphate buffered saline
PDI  = Polydispersity index
PLGA  = Poly(lactic-co-glycolic acid)
PVA  = Polyvinyl alcohol
SLNs  = Solid lipid nanoparticles
SC  = Stratum corneum
SEM  = Scanning electron microscopy
5α–Red  = 5α–Reductase
TEM  = Transmission electron microscopy
TPP  = Tripolyphosphate
UV  = Ultraviolet
Curriculum Vitae

Name: Mardiyanto
Date of birth: 10 March 1971
Place of birth: Padang, West Sumatra, Indonesia
Nationality: Indonesian

Education:

1999-2002: Master of Science (MSc) at the Department of Pharmacy, Faculty of Natural Science, Bandung Institute of Technology (ITB Bandung), Jalan Ganesha 10, Bandung, West Java, Indonesia. Thesis was entitled: "Enhancement the tetracycline production of Streptomyces aureofaciens".

1995-1996: Apoteker (Pharmacist) at Department of Pharmacy (professional program of pharmacist) Faculty of Natural Science, University of Andalas, Kampus Unand Limau Manis, Padang, Indonesia.

1990-1995: Bachelor of Science (BSc) at Department of Pharmacy, Faculty of Natural Science, University of Andalas, Kampus Unand Limau Manis, Padang, Indonesia. Best graduate from Faculty of Natural Science, University of Andalas, Padang, Indonesia

1990: Completed high school education at the Indonesian state high school SMAN 3, Jalan Gajah Mada, Padang, Indonesia.

Career Summary:

2009-Present: PhD student at Department of Pharmaceutical Nanotechnology, Saarland University, Saarbrücken, Germany.

2007-2008: Training on International Management Competence of Industrial Biotechnology, Germany.
2003- 2007: Indonesian government employer: Assistant lecturer at pharmaceutical study program, Faculty of Natural Science, Sriwijaya University, Inderalaya, Indonesia.

1998- 2000: Pharmacist at Apotek Bunda, Palembang, Indonesia


Publications:

Research articles:
- Mardiyanto, Clemens Tscheka, Wolfgang Bost, Marc Fournelle and Marc Schneider, “Photoacoustic Imaging of Model Hair Follicles Containing Magnetite-PLGA Nanoparticles”. 2013. In process of submission

Poster presentations:
- Mardiyanto, Clemens Tscheka, Marc Fournelle and Marc Schneider, “Photoacoustic Imaging of Model Hair Shaft” Inascon Conference. August. 2012.

Other Scientific Activities:

- Participation in the association of the Pharmacist in South Sumatera Indonesia, development of nature resources for traditional drugs supplying, 2003-2007.
- Participation in the Dikti development of research collaboration inter University in Indonesia. 2006-2007.
Grants:

- Indonesia Dikti Scholarship for a 3 years study at Saarland University for a PhD degree 2009-2012.
- International management competence on Industrial biotechnology, one year in Germany, June 2007-May 2008.
- Indonesia Dikti Scholarship for a 2 years study at Bandung Institute of Technology (ITB) Bandung, Indonesia for a MSc Degree 1999-2001.
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Mardiyanto