Characterization of SLN in o/w-emulsions

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ABSTRACT

Apart from their applications in the pharmaceutical and cosmetics industry, synthetic organic nanoparticles are now increasingly investigated to be used in food. Therefore only food grade materials are tolerable for the preparation of nanoparticles which have to be thoroughly characterized to ensure their safety.
To investigate synthetic organic nanoparticles within a model food matrix, solid lipid nanoparticles (SLN) were prepared and characterized by particle size and zeta potential as well as TEM measurements. Emulsions prepared with different emulsifiers (CTAB, Tween 20, SDS) were analyzed in the presence and absence of SLN in a similar manner. CLSM was used to localize the SLN within the emulsions and to visualize possible interactions with the lipid phase of the emulsions at their solubilization site.
The results indicate that the developed analytical systems are suitable to investigate interactions of SLN within model food systems such as emulsions. Differences between the emulsions in the presence and absence of SLN were analyzed via particle size as well as zeta potential measurements. Contrary to prior assumptions no significant difference between the controls and the SLN-enriched emulsions were detectable by imaging techniques.

1 Introduction

With the rising interest in nanoparticles and their application in food, the development of new methods and techniques for their characterization is necessary. Synthetic organic nanoparticles (NP) are primarily investigated as carriers of sensitive ingredients e.g. to increase their bioavailability or chemical stability. The detection and characterization of the isolated NP and NP within the food matrix is crucial to understand interactions with food components (Sekhon, 2010). Furthermore, it is important to determine possible consequences resulting from these interactions for the food system as well as in the gastrointestinal tract after consumption (Chaudry et al., 2008). The major problem of most characterization methods arises from the necessity to use food grade materials and the similar size ranges of the NP and the ambient food structures. Both make it difficult to detect engineered NP in complex food matrices. Therefore multiple methods have to be employed for a thorough characterization of NP (Tiede et al., 2008). After the characterization of the NP, they can be added to model food systems, which are subsequently characterized in the presence and absence of NP to determine possible interactions (Sapsford et al., 2011; Chaudry et al., 2008). Developed characterization methods then refer only to the studied food matrix with the selected NP and cannot necessarily be transferred to other systems. Furthermore, this indicates that it is not possible to screen real food systems for NP. Only with the prior knowledge of the usage of NP within a certain food, predictions concerning the interactions within the food matrix may be made.

Solid lipid nanoparticles (SLN) were developed in the pharmaceutical sector as an alternative carrier system to traditional colloidal systems such as liposomes, emulsions and polymeric nanoparticles (Radomska-Soukharev, 2011; Müller et al., 2011; Pardeike et al., 2011). SLN consist of a crystallized nanoemulsion with the dispersed phase being a solid carrier lipid. The loading capacity depends on the lipophilic nature of the bioactive compound as well as its interactions with the lipid phase. The choice of composition and utilized production method, influence the mean particle sizes of the SLN which normally range between 100-200 nm (Weiss et al., 2011a, b). SLN are used for the protection of sensitive active compounds, to increase the bioavailability of poorly soluble drugs, stabilize insoluble components, targeted drug delivery and for controlled drug release (Hu et al., 2011; Bunjes, 2011). Moreover, SLN are exceptionally stable, can be manufactured using biocompatible components and have a huge scaling-up potential (Radomska-Soukharev, 2011; Müller et al., 2011; Jores et al., 2011; Wissing et al., 2011). As a consequence of these properties, SLN also offer enormous possibilities for applications in food as a nontoxic carrier system. For instance, SLN can be used for the encapsulation of sensitive bioactive compounds such as phytochemicals or vitamins (Mehnert and Mäder, 2011).

In this study, emulsions were used as a basic model system for the investigation of possible interactions with SLN. Therefore, they were solely prepared using a lipid and a liquid phase as well as the addition of differently charged emulsifiers. CTAB as a cationic, SDS as an anionic and Tween 20 as a non-ionic surfactant were used to simulate different environments. Emulsifiers accumulate at the o/w-interface of an emulsion, which thereby acquires a certain charge. The used SLN, which were added to the emulsions, had a negative surface charge. Hence a working hypothesis was established, that the SLN would interact with the differently charged lipid phase of the emulsions depending on their surface charge. For instance SLN would be repelled by the negative surface charge of the SDS molecules at the o/w-interface in the emulsions and attracted by the positive surface charge of the CTAB molecules. The latter possibly leads to attachment of the SLN to the o/w-interface similar to Pickering emulsions.

2 Materials and Methods

2.1 Materials

Glyceryl tristearate, sodium dodecyl sulfate (SDS), Tween 20, cetyltrimethylammoniumbromid (CTAB), Coumarin 6 and Nile Red were purchased from Sigma Aldrich (Steinheim, Germany). Acetic acid was purchased from Roth (Karlsruhe, Germany) and sodium acetate trihydrate from Merck (Darmstadt, Germany). MCT-oil was purchased from Schumann & Sohn (Karlsruhe, Germany) and gelatine was acquired from RUF (Quakenburg, Germany). Lecithin (Epikuron 100) was a gift from Cargill (Hamburg, Germany), Ryoto sugar ester S1670 was kindly provided by Harke Food Tech. (Mühlheim, Germany)

2.2 Preparation of SLN

SLN were produced by a hot emulsification method using glyceryl tristearate as the matrix lipid. The lipid phase, together with the emulsifier (Epikuron), was heated 5-10 °C above its melting point for 30 min. After adding a previously heated aqueous solution of S1670, a pre-emulsion was obtained by mixing both phases together. The nanoparticles were formed using ultrasound (Sonopuls HD 3100 with sonication probe VS 70T, Bandelin, Germany) for 30 min with an amplitude of 75 %. Subsequently, the hot nanoemulsion was mixed with a third surfactant solution (Tween 20) ensuring the stability of the SLN and cooled down in a water bath to room temperature. The SLN were dialyzed for 24 h against sodium acetate-acetic acid-buffer (pH 5.0, 0.2 M) to remove any excess surfactant.

2.3 Preparation of emulsions

Oil-in-water-emulsions were prepared, consisting of 10 wt% MCT-oil and 90 wt% sodium acetate-acetic acid-buffer solution (pH 5.0, 0.2 M) with either 10 mM SDS or CTAB or 1.2 mM Tween 20 as emulsifier. The emulsions were produced by weighting 15 g MCT-oil into a glass bottle, to which 45 ml emulsifier solution was added. Prior to sonication the two phases were stirred at 400 rpm for 10 min. The pre-emulsion was sonicated (Sonopuls HD 3100 with sonication probe VS 70T, Bandelin, Germany) at an amplitude of 40 % for 30 s. Then 90 ml of a sodium acetate-acetic acid-buffer solution was added gradually over 60 s and after that the emulsion sonicated for additional 60 s.

A portion of each emulsion (SDS, Tween 20, CTAB) was mixed with SLN in a ratio of 10:1. Emulsions with and without the addition of SLN were prepared in triplicate and stored at 25 °C in the dark for three weeks.

2.4 Particle size and zeta potential measurements

The mean hydrodynamic diameter, also referred to as Z-Average and the polydispersity index (PI) of the SLN was measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Laser diffractometry (Mastersizer 2000E, Malvern, UK) was used to assess the size distribution of the oil droplets of the emulsions. Control and SLN-enriched emulsions were diluted prior to the measurements with Milli-Q water.

Zeta potential measurements were performed using the Malvern Zetasizer Nano ZS. The SLN and emulsions were diluted with Milli-Q water to adjust the conductivity to 50 µS/cm (Müller, 1996). Particle size and Zeta potential measurements were performed on the day of production and weekly during storage for three weeks.

2.5 Characterization of SLN via TEM

Samples of SLN were examined by freeze-fracture transmission electron microscopy (TEM) using a Philips EM 301 transmission electron microscope operated at 60 kV. A droplet of each sample was fixed via shock freezing and was freeze-fractured at -120 °C without etching using a Balzers BA 360 M unit (Balzers,

Lichtenstein). Samples prepared accordingly were replicated by application of Pt/C and C via electron-gun evaporation (Schrader, 1997).

2.6 Confocal laser scanning microscopy of emulsions

The differently prepared emulsions were visualized by confocal laser scanning microscopy (CLSM) using al LSM 510 META (Carl Zeiss Microscope Systems, Jena, Germany). For imaging of the lipid phase of the emulsions, 0.1 g of a 0.065 % Nile Red in MCT-oil solution was added to the oil phase prior to emulsification. To image the SLN a total of 0.025 % Coumarin 6 from a stock solution was added to the hot pre-emulsion and dissolved by stirring for at least 30 min, before cooling down the nanoemulsion to room temperature.

Prior to the imaging the emulsions were diluted 1:10 with water. The liquid samples were mixed with 1 wt% gelatine and poured into a shallow square-cut mould. The resulting blocks were cut into 0.5 mm slices and examined under the microscope. Nile Red molecules were excited at a wave length of 561 nm and the fluorescence emission intensity was collected over 615-647 nm. Coumarin 6 was excited at a wave length of 488 nm and the fluorescence emission intensity collected at 505-550 nm. The optical magnification was achieved by a Plan-Apochromat 63x/1.4 Oil DIC objective using Immersol 518F (Zeiss, Oberkochen, Germany).

3 Results

3.1 Particle size and zeta potential

Mean particle sizes and zeta potentials of SLN and of the different control and SLN-enriched emulsions were measured over a period of three weeks (Tab. 1). The particle sizes of the emulsions and SLN are given as the Sauter mean diameter (d_{32}) and as the Z-Average respectively. No alteration in particle size could be observed during storage of two weeks. However, afterwards a creaming of the control emulsions could be observed visually and via the analytical data. Control emulsions began to separate, indicated by a creamy top layer and an increasingly clear bottom phase. This was clearly visible in the size measurements, where the mean diameter of the particle sizes of the control emulsions increased. The addition of SLN stabilized the emulsion droplet sizes, showing no alteration in particle size distribution.

Table 1 Selected data of the particle size and zeta potential measurements of SLN and emulsions. Particle size is given as the Sauter mean diameter (d_{32}) in µm, the particle size of SLN is given as Z-Average.

	Size [µm]			Zeta potential [mV]		
	Day 0	**Week 2**	**Week 3**	**Day 0**	**Week 2**	**Week 3**
SDS	1.58±0.02	1.53±0.02	2.78±0.04	-110±3	-102±6	-104±5
SDS + SLN	1.68±0.05	1.67±0.02	1.68±0.03	-92±4	-91±3	-89±4
Tween 20	1.64±0.04	1.67±0.01	1.91±0.02	-13±1	-23±1	-8±1
Tween 20 + SLN	1.51±0.19	1.51±0.03	1.52±0.02	-33±5	-40±3	-37±3
CTAB	1.64±0.01	1.62±0.01	1.64±0.02	106±5	107±5	101±4
CTAB + SLN	1.59±0.03	1.62±0.06	1.57±0.02	90±3	90±2	89±1
SLN	0.163±0.001	0.167±0.01	0.163±0.002	-39±1	-39±1	-38±1

The zeta potentials of the different emulsions caused by the utilized emulsifier, i.e. emulsions containing the anionic SDS, cationic CTAB or non-ionic Tween 20 had zeta potentials of -110 mV, 106 mV and -13 mV, respectively. The SLN had a zeta potential of -39mV, which did not change during the three week storage. SLN-enriched emulsions prepared with ionic surfactants exhibited a shift in the zeta potential of roughly 15-20 mV compared to the respective control emulsions. While the zeta potential of SDS emulsions increased, the zeta potential of CTAB emulsion decreased by the amount mentioned. The zeta potential in the SLN-enriched Tween 20 emulsions decreased up to 30 mV compared to the control emulsions to approximately the zeta potential of SLN.

3.2 Imaging of SLN and emulsions

TEM revealed that the SLN are platelet shaped and consist of different layers (Figure 1). In the side view they look like needles or rods with a thickness of about 50 nm.

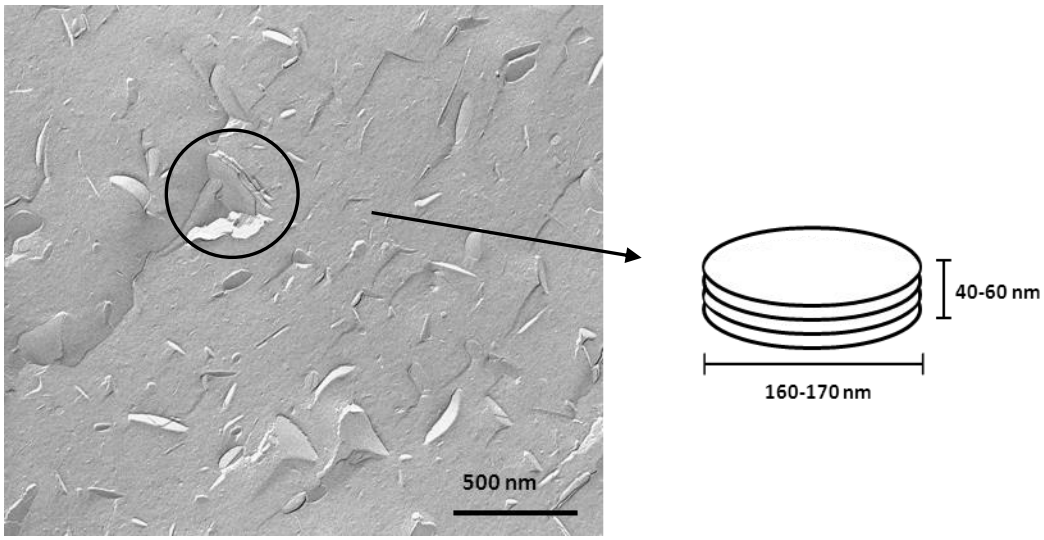

Fig. 1 TEM picture of SLN on the left side, with a schematic view of the examined structure on the right.

Confocal laser scanning microscopy was used to investigate the interactions of SLN with the oil droplets of the different emulsions. The size distribution of the oil droplets within the emulsions was in accordance with the previously obtained particle size measurements. No visual difference between the emulsifiers could be determined. As an example, a SDS emulsion containing SLN is depicted in Fig. 2. In part a) of the image, an overlay of the pictures of both dyes is shown that were used to stain the lipid phase of the emulsion or the SLN respectively. Images b) and c) represent the individual images of the oil droplets and SLN. These images indicate that both the SLN and the oil droplets have the same location. However, some oil droplets differ in intensities concerning the Coumarin 6 emission.

Images were taken right after production and at intervals up to two months. Although changes in particle sizes were apparent, indication for changes in the location of the SLN did not appear.

4 Discussion

The analysis of particle size distributions via dynamic and static light scattering are well-established methods (Peters et al., 2011) and their applicability for the assessment of inorganic nanoparticles has been analyzed and reviewed (Murdock et al., 2008; Brar and Verma, 2011). Those methods can also be used to study the particle size distribution and stability of organic structures such as emulsions and SLN.

However, concerning the mean particle size of SLN, the measurement via dynamic light scattering has some drawbacks, most of all spheres being the basis for size calculations. As described in Figure 1, SLN are platelet shaped and therefore significantly differ from a sphere. Furthermore, due to their shape, SLN have a larger surface area than spheres, which can result in increased interactions with surrounding matter (Mehnert and Mäder, 2001). To acquire accurate data of the shape of nanoparticles, imaging techniques such as electron microscopy are indispensable.

Static light scattering serves as a sufficient tool for the particle size measurement of emulsions. Predictions about the stability are indicated by the width of the particle size distribution and its development over time. By comparing emulsions with and without the addition of SLN, the stabilizing effect of the SLN on the emulsions can be observed.

Measurements of the zeta potential provide further insights into the utilized emulsifier as well as the possible interactions with SLN. Zeta potentials of +/-30 mV indicate stable particles (Müller, 1996). Despite the highly zeta potentials obtained with ionic emulsifiers, the emulsions showed signs of instability after two weeks. The zeta potential of all emulsions was influenced by the addition of SLN. Although the zeta potential of the ionic surfactants was affected similarly, differences in the partitioning of the emulsifiers could also influence the interactions with SLN (Oehlke et al., 2008). Non-ionic surfactants are slightly charged depending on the surrounding medium (McClements et al., 2011). Therefore, the influence of the addition of SLN on Tween 20 emulsions was particularly evident. The shift of the barely negative zeta potential of the control emulsion to the zeta potential of the SLN upon addition is an indicator of their interaction. Reasons for this could be the

adsorption of SLN to the o/w-interface similar to Pickering emulsions (Gupta and Rousseau, 2012) or the uptake into the oil droplet as e.g. displayed by some antioxidants (Mei et al., 1999). It may be assumed that different sub-solubilization sites of antioxidants in micelles and emulsion interfaces that are generated by the emulsifier investigated (Heins et al., 2007a, b) may also lead to a different location of SLN in the non-ionic relative to the ionic emulsion interface. This may explain the alteration of zeta potential to a different extent comparing ionic and non-ionic emulsions with SLN added.

Imaging techniques are essential for the analysis of nanoparticles in order to determine parameters such as particle shape or particle behavior (e. g. agglomeration). Furthermore, imaging can be used to visualize possible adsorption of the nanoparticles with the surrounding matrix as displayed in Pickering emulsions. The collected CLSM-images confirm interactions of SLN with the emulsions' lipid phase, because both are located in the same area. However, besides the location of both fluorescent dyes, no information on the condition of the SLN can be obtained. Fluctuations in fluorescence intensities of the lipid phase indicate different extents of interactions with the SLN. The results suggest differences in the uptake or adsorption of SLN to the lipid phase. Whether these are due to charge differences because of the different surfactants is currently under investigation, e.g. using EPR spectroscopy.

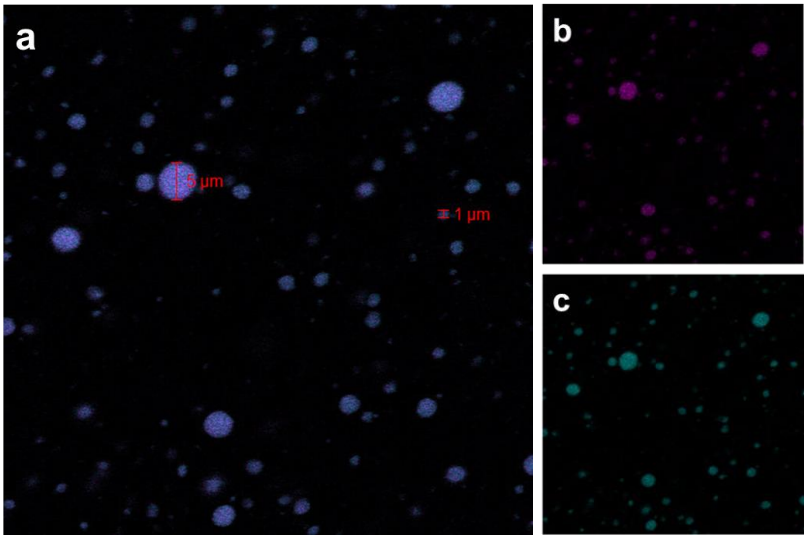

Fig. 2 CLSM-images of a SDS-emulsion containing SLN. The lipid phase was stained with Nile Red, the SLN with Coumarin 6. a) Overlay of both images, b) image of Nile Red-stained emulsion oil droplets, c) Coumarin 6-stained SLN.

5 Conclusions

The presented methods provide a first approach to study the interactions of SLN with o/w-emulsions. A stabilizing effect of the SLN on emulsions could be shown by measurements of the particle size distribution during storage. Zeta potential measurements revealed interactions of SLN with the o/w-interface of all emulsions regardless of the emulsifier used. Additionally, imaging via CLSM displayed that the site of SLN equate to the lipid phase of the emulsions. Because both ionic surfactants were influenced by the addition of SLN to the same extend, the interactions with the o/w-interface are not influenced by the surface charge. The previous assumptions of the formation of Pickering emulsions as well as the repulsion depending on surfactant charge were disproven.

However, it has to be pointed out, that the model emulsions under study did not contain any additional ingredients usually present in food. Hence, interactions with macromolecules such as proteins and carbohydrates or influences of food structure were neglected. In regard to the question whether the zeta potential is influenced by different sub-locations within the lipid phase depending on the used emulsifier, spectroscopic methods such as EPR can be applied. Furthermore, TEM can be used for a visual analysis of the o/w-interface in emulsions.

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