Thiolation of non-ionic surfactants for the development of lipid-based mucoadhesive drug delivery systems

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ABSTRACT

The aim of this study was to develop thiolated self-emulsifying drug delivery systems (SEDDS) and nanostructured lipid carriers (NLCs) with improved mucoadhesive properties. Two non-ionic surfactants bearing a short and long PEG chain, namely polyoxyethylene (10) stearyl ether (PSE_{10}) and polyoxyethylene (100) stearyl ether (PSE_{100}), were thiolated for the first time by substituting the terminal hydroxyl group with a thiol group. The synthesis was confirmed by FT-IR, NMR and Ellman's test. SEDDS and NLCs containing these thiolated compounds were investigated for size, polydispersity index (PDI) and ζ potential. Subsequently, mucus diffusion studies, rheological evaluations after mixing the nanocarriers with mucus and mucoadhesion studies on porcine intestinal mucosa were performed. All nanocarriers had a size less than 250 nm, a maximum PDI of 0.3 and a ζ potential < -9.0 mV. Mucus diffusion studies resulted in the rank order of increasing diffusivity: PSE_{10}-SH < PSE_{100}-SH < PSE_{10}-OH < PSE_{100}-OH for NLCs and PSE_{10}-OH < PSE_{100}-OH < PSE_{100}-SH < PSE_{10}-SH for SEDDS. The mucoadhesive properties and increase in viscosity of SEDDS and NLCs ranked: PSE_{100}-OH < PSE_{10}-OH < PSE_{100}-SH < PSE_{10}-SH. In addition, the short chain PSE_{10}-SH showed higher mucus interactions than the long chain PSE_{100}-SH for both SEDDS and NLCs. The thiolated PSE surfactants appeared to be promising excipients for the design of highly mucoadhesive drug delivery systems.

Keywords: Lipid Based Drug Delivery Systems; Non-ionic Surfactants; Mucoadhesion; Mucodiffusion; Thiomers.

1. INTRODUCTION

Drug administration via mucosal membranes is preferred over other routes, as it shows higher compliance and acceptance by patients. However, hurdles such as poor solubility of drugs and consequently low bioavailability have not yet been entirely mastered. To overcome these obstacles lipid-based formulations such as self-emulsifying drug delivery systems (SEDDS) and nanostructured lipid carriers (NLCs) have been developed. Besides the enhanced drug loading capacity, both carrier systems exhibit additional advantages that make them even more attractive for researchers in academia and industry. Compared to other lipid-based formulation, SEDDS are simple to produce and can be easily scaled up by the manufacturer [1,2]. In addition, SEDDS overcome the stability problems of other carrier systems by avoiding agglomeration during storage, as they are only formed in the gastrointestinal tract after oral administration. NLCs have been developed to avoid drug excretion during storage of nanocarriers [3]. In comparison to solid lipid nanoparticles, the additional liquid lipid inside NLCs improves the loading capacity for drugs and causes a stable incorporation of them inside the carrier [4]. Furthermore, stable NLCs can be formed by smaller amounts of surfactants lowering cytotoxic effects [3]. Due to the short residence time in the gastrointestinal tract, however, the potential of SEDDS and NLCs is limited after oral administration [5]. A promising approach to address this shortcoming is the attachment of thiol groups on the surface of these nanocarriers to increase their mucoadhesive properties [6]. Thiolated polymers, called thiomers, have shown great potential to improve mucoadhesion in numerous studies [7–11]. Unthiolated nanocarriers can only adhere to the mucosa by weak electrostatic interactions such as hydrogen bonding and van der Waals interactions [12]. In comparison, thiomers can form strong covalent bonds due to disulfide bond formation between cysteines of the cysteine-rich mucosa and free thiols on the surface of the nanocarrier [13,14]. Along the improved mucoadhesive properties, thiomers show also permeation enhancing as well as enzyme and efflux pump inhibitory properties [15].

There are various approaches to prepare thiolated nanocarriers. One common method is the coating of already formed nanocarriers with thiolated excipients [14,16]. Since such coatings can get lost in the harsh gastrointestinal environment, thiolated excipients have to be properly attached on the surface of the nanocarrier [17]. This can be realized by using thiolated surfactants whose lipophilic tails are anchored in the oily phase of nanocarriers. Non-ionic surfactants such as PEGylated surfactants are often used due to their high emulsifying properties [18]. Moreover, the inert PEG corona enables additional advantages for the nanocarrier, such as increased mucus permeability and reduced enzymatic degradation [19].

However, the potential of these PEGylated lipid-based nanocarriers is not yet fully exploited due to missing mucoadhesive properties. To further improve the advantages of PEGylated nanocarriers, the aim of this study was to develop SEDDS and NLCs equipped with thiolated PEGylated surfactants to form nanocarriers exhibiting high mucoadhesion. For this purpose, surfactants bearing a short and long PEG chain, as illustrated in Fig. 1, were chosen since the chain length of PEG can have an influence on its conformation and thus its behavior in biological systems [20]. Polyoxyethylene (10) stearyl ether and polyoxyethylene (100) stearyl ether were thiolated for the first time by substituting the terminal hydroxyl group with a thiol group. The thiolated surfactants were characterized by FT-IR, NMR and Ellman's test. The thiolated SEDDS and NLCs were evaluated in terms of size, polydispersity index (PDI) and ζ potential. In addition, the mucus interaction properties of nanocarriers were investigated in vitro by mucus diffusion studies, rheological evaluations after mixing the nanocarriers with mucus and mucoadhesion studies on porcine intestinal mucosa.

2. MATERIALS AND METHODS

2.1 Materials

Cremophor EL (polyethoxylated-35 castor oil, hydrophilic-lipophilic balance (HLB = 13)), ethanol, methanol, chloroform, eugenol, benzyl alcohol (BA), polyoxyethylene (10) stearyl ether (PSE_{10}-OH, HLB =12), polyoxyethylene (100) stearyl ether (PSE_{100}-OH, HLB=18), Triton-X 100, L-cysteine, lithium bromide, N-bromosuccinimide (NBS), thiourea, triphenylphosphine, diethyl ether and sodium sulphate were purchased from Sigma-Aldrich Italia SRL (Milano, Italy). Cetyl palmitate (> 98%) (HLB = 9) was purchased from Thermo Fisher Scientific Italy (Rodano, Italy). Pluronic F68 and Lumogen F Red 300 (LFR) were purchased from BASF (Ludwigshafen, Germany). Sodium borohydride, hydrochloric acid solution, silica gel 60 (0.040-0.063 mm), oleic acid and oleyl alcohol were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2 Synthesis of thiolated PSE_{10}/PSE_{100}

Thiolated PSE (PSE_{10}-SH and PSE_{100}-SH) was obtained through two consecutive synthesis steps. Both steps were performed under inert conditions by introducing N_2 gas into the reaction chamber. The NBS and Ph_3P reagents were dried before use in a desiccator under reduced pressure and in the presence of 4 Å molecular sieves. In the first step of the synthesis, the starting compounds were brominated. In detail, NBS (1.1 mmol) and PPh_3 (1.0 mmol) were dissolved in 5 mL of CH_2Cl_2. PSE_{10}-OH or PSE_{100}-OH (1.0 mmol) were added to the resulting solution. The solution was left under

stirring overnight at a constant temperature of 40 °C. After cooling down to RT, an equal volume of diethyl ether was added to the resulting solution to precipitate Ph_3PO. The solution was therefore kept at 0 °C overnight and then the obtained precipitate was removed by centrifugation (SL 16R Centrifuge, Thermo Scientific, USA) at 13000 rpm and 4 °C for 15 minutes. The resulting solution was dried under vacuum using Rotavapor R-200 (BUCHI Italia Srl), obtaining brominated compounds PSE_{10}-Br and PSE_{100}-Br as a clear oil. In the second step of the synthesis, bromine moieties (-Br) were replaced by thiol groups (-SH) through a nucleophilic substitution reaction with thiourea. In detail, the previously obtained brominated product was dissolved in 2 ml of DMF. Then, 5 mL of thiourea solution (5.0 mg/mL) in DMF were added to this solution. The reaction was left under stirring overnight at a constant temperature of 90 °C and under reflux conditions. Subsequently, a vacuum distillation was performed to remove DMF. Progress of the reaction was followed by TLC (Silica 60 F254 gel, Merck, Germany). Further purification was performed by column chromatography (stationary phase: Silica gel 60, 230-400 mesh, Merck, Germany; mobile phase: acetone/dichloromethane 1/1). Finally, the appropriate fractions were dried in a rotavapor to obtain a solid thiolated product (PSE_{10}-SH and PSE_{100}-SH) of waxy consistency.

<< Insert Fig. 1 >>

2.3 Thiolated compounds characterization with FT-IR, ^{1}H-NMR and Ellman's Test

PSE_{10}-OH, PSE_{100}-OH, PSE_{10}-Br, PSE_{100}-Br, PSE_{10}-SH and PSE_{100}-SH were characterized by FT-IR and ^{1}H-NMR. FT-IR spectra were analyzed by FT-IR 1600 Perkin Elmer. Data were acquired between 4000 cm^{-1} and 450 cm^{-1}. ^{1}H-NMR spectra were obtained by an Agilent VNMRS 500 MHz spectrometer using $CDCl_3$ as solvent. Chemical shifts were referenced by using the solvent residual peak of $CDCl_3$ at 7.26 ppm.

PSE_{10}-Br/PSE_{100}-Br: Yield 80% w/w, (0.480 g) for PSE_{10} and yield 60% w/w, (0.360 g) for PSE_{100}. FT-IR (KBr): 3500, 2900, 1465, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 3.72 (t, 3H, CH_3), 4.00-3.50 (m, CH_2), 1.50 (m, CH_2), 1.30-1.20 (m, CH_2), 0.75 (t, CH_3) ppm.

PSE_{10}-SH/PSE_{100}-SH: Yield 85% w/w (0.408 g) for PSE_{10} and yield 70% w/w, (0.250 g) for PSE_{100}. FT-IR (KBr): 3430, 2850, 1450, 1100, 1060, 630 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 3.55 (t, 3H, CH_3), 3.80-3.60 (m, CH_2), 1.90-1.55 (m, CH_2), 1.40-1.25 (m, CH_2), 0.75 (t, CH_3) ppm.

Ellman's tests was performed to confirm thiolation of the surfactants [21], using a standard curve prepared with increasing concentration of cysteine. A solution of 1 mg of the thiolated surfactant dissolved in 500 μL of 1.0 M PBS pH 8.0 was added to 500 μL of fresh Ellman’s reagent and incubated for 120 minutes at RT in a light-protected box. The mixture was centrifuged in an Eppendorf Minispin at 13,000 rpm for 5 minutes. Subsequently, 100 μL of each obtained sample were transferred to a Spark multifunctional microplate reader (Tecan infinite M200 spectrophotometer, Tecan, Grödig, Austria) for evaluation of absorbance at 450 nm. All experiments were performed in triplicate.

2.4 Preparation of NLCs

NLCs were prepared by a high-temperature oil-in-water homogenization process following a previous slightly modified method [22]. Briefly, 80 mg of cetyl palmitate and 20 mg of oleic acid were co-dissolved in chloroform (1.0 mL) and methanol (0.5 mL) to form the organic phase. This phase was added dropwise to a 5 mL solution of ultrapure water containing Pluronic F68 (1% w/v) at 65 °C and immediately sonicated for 15 minutes with the UP-200 H ultrasonic processor (dr.Hielscher Ultrasonics GmbH, Teltow, Germany). PSE was incorporated into the organic phase, specifically 12 mg of PSE_{10}-OH or PSE_{100}-OH or PSE_{10}-SH or PSE_{100}-SH were added generating $NLC_{PSE10-OH}$, $NLC_{PSE100-OH}$, $NLC_{PSE10-SH}$ and $NLC_{PSE100-SH}$, respectively (Table 1). The NLC preparations were left at room temperature for two hours to permit the complete evaporation of the organic solvents and then, they were maintained at 4 °C for 15 min to allow the NLCs formation. To remove any surfactant, solvent residuals and non-encapsulated drug, the produced NLCs were carefully washed using ultrapure water and centrifugal concentrators (Centricon Centriplus YM100) at 800 g for 1 hour at 4 °C. All the nanoformulations were kept in ultrapure water at 4 °C.

<< Insert Table 1 >>

2.5 Preparation of SEDDS

SEDDS were prepared by mixing oil, emulsifier, and cosolvents/cosurfactants in the ratios listed below. PSE_{10}-OH, PSE_{100}-OH, PSE_{10}-SH or PSE_{100}-SH were added to the mixture generating $SEDDS_{PSE10-OH}$, $SEDDS_{PSE100-OH}$, $SEDDS_{PSE10-SH}$ and $SEDDS_{PSE100-SH}$, respectively. The mixtures were subsequently stirred at 2000 rpm and 37°C for 30 min in a thermomixer (Eppendorf, Hamburg, Germany) in order to obtain clear solutions. Resulting SEDDS were dispersed in distilled water in a ratio of 1:100 by stirring at 50 rpm.

SEDDS excipient composition in percentage (% v/v): eugenol 15% as cosolvent/cosurfactant; benzyl alcohol 15 % and ethanol 1.8 % as cosolvents; Cremophor EL 20 % as surfactant; PSE_{10}-SH and PSE_{100}-SH 18.2 % as mucoadhesive surfactant; oleyl alcohol 30 % as oil phase.

2.6 Preparation of NLCs and SEDDS labeled with LFR fluorescent

To investigate the mucoadhesive properties of the samples, NLCs and SEDDS were loaded with 0.1% (w/w) of an LFR fluorescent probe. LFR is soluble in DMF (70 g/L, at 20°C) and can be measured via fluorescence at λ_{ex} = 570 nm and λ_{em} = 610 nm. Labeled SEDDS were dispersed in distilled water in a ratio of 1:100 by stirring at 50 rpm.

2.7 Dynamic Light Scattering (DLS) and ζ potential

The mean hydrodynamic diameter, size distribution, and ζ potential values of the NLCs and SEDDS were determined by using the Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK (DTS 5.00). Size and size distribution were measured via dynamic light scattering (DLS) at room temperature after sample dilution in demineralized water. Size distribution was described in terms of polydispersity index (PDI) and the average particle size was reported as intensity mean diameter. The ζ potential measurements were carried out by using a laser Doppler velocimetry (LDV) after sample dilution in freshly prepared aqueous KCl solution (1 mM).

2.8 Purification of small intestinal mucus

Porcine small intestine was a kind gift of a local slaughterhouse (Bari, Italy). Small intestine obtained from freshly slaughtered pigs was cut into 10 cm long pieces. Intestines containing chyme were discarded. The mucus was gently removed from the mucosa with a finger and the collection was cooled on ice. Afterwards, mucus was purified for further use [23]. For this purpose, mucus was mixed with 0.1 M NaCl in a concentration of 20 % (w/v). The mixture was stirred on ice for one hour and centrifuged at 10,400 g and 10 °C for 2 hours. Supernatant and granular material on the bottom of the centrifuged tubes were wasted. The purification procedure was repeated twice. The purified mucus was homogenized and then frozen at -20 °C until further use.

2.9 Mucus diffusion studies

Diffusion properties of NLCs ($NLC_{PSE10-OH}$, $NLC_{PSE100-OH}$, $NLC_{PSE10-SH}$, $NLC_{PSE100-SH}$) and SEDDS ($SEDDS_{PSE10-OH}$, $SEDDS_{PSE100-OH}$, $SEDDS_{PSE10-SH}$ and $SEDDS_{PSE100-SH}$) samples were evaluated by a rotating tube method, as previously described [14]. Silicon tubes used in the study were obtained from

Lactan (Graz, Austria). Briefly, silicone tubes with an inner diameter of 30 mm were cut into pieces of 4 cm in length and filled with 150 µL of purified intestinal mucus using a 1.0 mL syringe. After adding the mucus, the tube was sealed with parafilm on one end. After deposition of 50 µL of NLCs or SEDDS dispersion (1:100 in distilled water) on the top of the mucus, the tube was closed with parafilm on the other end. The test tubes were rotated at 37 °C and 50 rpm for 24 hours under light protection and then frozen at -80 °C. The obtained frozen tubes were cut into 10 segments of 2 mm in length. Each frozen slice was treated with 500 µL of DMF to extract LFR. Therefore, the samples were incubated in a horizontal shaker for 2 hours at 150 rpm and centrifuged thereafter for 5 min at 13,400 rpm using a MiniSpin Centrifuge (Eppendorf, Hamburg, Germany). The fluorescence intensity of 100 µL of each sample was measured at an excitation wavelength of 570 nm and an emission wavelength of 610 nm using a Tecan microplate reader. As 100% control, the theoretical initial amount of LFR present in donor compartment was determined. Percentage of LFR permeated was calculated by percent ratio of LFR amount in each slice of mucus to 100% control.

2.10 Rheological investigations

Interactions of the NLCs and SEDDS with mucus were investigated by a rheological measurement [14]. Therefore, 500 mg of purified mucus was gently homogenised with a spatula with 500 µL of undiluted NLCs and SEDDS previously diluted to the same concentration. The mixture was incubated for 4 hours at 37 °C in an incubator and then transferred to the lower plate of a Haake Mars plate-plate Rheometer (Thermo Scientific, Vienna, Austria). A strain sweep measurement was conducted at a frequency of 1 Hz and a shear range of 0.01 Pa to 50.0 Pa. Additionally, a frequency sweep measurement was performed within the linear viscoelastic region at a shear rate of 0.1 Pa and a frequency range of 0.1 Hz to 20.0 Hz. The temperature of the rheometer was set to 37 °C during the whole study. Mucus only mixed with PBS served as control.

2.11 *In vitro* evaluation of mucoadhesive properties

In vitro mucoadhesion studies were performed on porcine intestinal mucosa (local butchery). NLCs ($NLC_{PSE10\text{-}OH}$, $NLC_{PSE100\text{-}OH}$, $NLC_{PSE10\text{-}SH}$, $NLC_{PSE100\text{-}SH}$) and SEDDS ($SEDDS_{PSE10\text{-}OH}$, $SEDDS_{PSE100\text{-}OH}$, $SEDDS_{PSE10\text{-}SH}$ and $SEDDS_{PSE100\text{-}SH}$) samples were evaluated regarding their mucoadhesive properties by a previously described method with some modifications [24]. First, porcine intestinal mucosa was opened lengthwise, cut into 5 x 2 cm, and attached to half-cut 50 mL falcon tubes by double-sided adhesive tape. Two different solutions containing 100 µL of NLCs and 100 µL of dispersed SEDDS (1:100 in distilled water) loaded with 0.1 % (w/v) LFR were added to the intestinal mucosa and incubated horizontally at 37 °C for 10 minutes. Afterward, the mucosa was orientated in

an incubator at 37°C with 100 % relative humidity at a 45° angle and rinsed for 2 hours using a peristaltic pump (Ismatec IPC 8, Cole-Parmer GmbH, Wertheim, Germany) with 1.0 mL/min flow rate with 50 mM phosphate buffer pH 7.4. The wash-off was collected every 10 minutes in 50 mL falcon tubes. After 2 hours, the mucosa was transferred to 10 mL DMF to extract the remaining LFR. The fluorescence intensity of the wash-off and extracted tissue was measured photometrically at an excitation wavelength of 570 nm and an emission wavelength of 610 nm. The percentage of remaining NLCs and SEDDS on mucosa was calculated for each intestinal mucosa extract.

2.12 Statistical data analysis

Statistical data were analyzed by one-way ANOVA in combination with Bonferroni post-test to analyze the significance of differences between means of more than two groups and two-way ANOVA with Bonferroni post-test. Calculations were performed with GraphPad Prism 5.01. The minimum significance level was set to $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Synthesis of PSE_{10}- SH /PSE_{100} - SH

PSE_{10}-SH/PSE_{100}-SH were obtained through substitution of hydroxyl groups with bromine moieties, followed by nucleophilic substitution with thiourea, as schematized in Fig. 1. Bromination makes the molecule highly reactive towards a wide range of nucleophilic reactions [21]. The reaction was conducted with direct bromination of hydroxyl groups (-OH) using NBS as brominating agent in combination with the dehydrating agent PPh_3. The reaction temperature was kept low to avoid the degradation of any reagents. The reaction conditions were initially set up by varying the concentration of the reagents and the type of solvent (data not shown) in order to supress the formation of secondary products and to increase the yield. In particular, the use of DMF as a reaction solvent resulted in incomplete bromination. Efficacy of purification including the removal of the main by-product Ph_3PO was confirmed by ^{1}H-NMR spectra (absence of aromatic peaks between 7.0 – 8.0 ppm). In the second step of the reaction with thiourea, the alkyl halide was converted to a thiol. Thiourea reacts with the alkyl halides to form an isothiuronium salt. Subsequent hydrolysis of this salt under the same reaction conditions results in a thiol group [25]. Finally, the purification by flash-chromatography yielded the desired product in high yield. The final product obtained was an odorless waxy solid that had a lacteous white color.

3.2 Characterization via FT-IR, ^{1}H-NMR and Ellman's test

FT-IR spectra were used to confirm the presence of the thiol group in the PSE_{10}-SH/PSE_{100}-SH through an investigation of the variation in peak position (in Fig. 2 are shown the spectra related to the samples PSE_{10}-OH, PSE_{10}-Br and PSE_{10}-SH). Unmodified PSE_{10}-OH/PSE_{100}-OH showed a broad characteristic peak around 3450 cm^{-1} related to the terminal -OH group (νOH) of the surfactants and intense peaks around 2900 cm^{-1} and 2850 cm^{-1} related to the CH stretching (νCH_2) and a broad peak around 1110 cm^{-1} associated to the C-OH and C-O-C stretching. Additional peaks can be observed in the spectra of PSE_{10}-SH/PSE_{100}-SH at about 620 cm^{-1}, relating to the stretching vibrations of the C-S group. These peaks are missing in the spectra of the unmodified products confirming successful thiolation. The ^{1}H-NMR spectra also confirmed the molecular structure of PSE_{10}-Br/PSE_{100}-Br and PSE_{10}-SH/PSE_{100}-SH (in Fig. 3 are shown the spectra related to the samples PSE_{10}-OH, PSE_{10}-Br and PSE_{10}-SH). The proton signal at 3.27 ppm, attributed to CH_2OH in the spectrum of the starting compound, turned out to be completely shifted downfield to 3.78 ppm after the bromination reaction, confirming the substitution of -OH group by -Br. In the spectrum of PSE_{10}-SH/PSE_{100}-SH, these methylene protons were shifted to a higher field at 3.55 ppm confirming the substitution of the -Br group by -SH. A peak at 6.52 ppm, corresponding to the signal of the -SH group, provided further evidence for the thiolation of the final product. The substitution of the OH group by a -SH group was also confirmed via Ellman's test.

<< Insert Fig. 2 >>

<< Insert Fig. 3 >>

3.3 Preparation and characterization of NLCs

For the formulation of NLCs, cetyl palmitate was used as a solid lipid and oleic acid as a liquid lipid. NLCs were prepared by the hot homogenization technique, carried out at temperatures above the melting point of lipids. In order to improve the mucoadhesive properties of NLC, thiolated PSE_{10} and PSE_{100} were added to NLCs. The size, PDI, and ζ potential of the resulting thiolated NLCs were evaluated as shown in Table 2. All preparations of NLCs exhibited a size below 150 nm. The particle size of NLCs plays an essential role for their mucus permeating properties. In fact, nanoparticles

smaller than 300 nm were shown to be more effective in permeating the intestinal mucus layer [26]. The size of NLCs having been designed within this study seems to be appropriate. The PDI was found in a range of 0.167-0.194 in all the preparations, indicating the homogeneous distribution of nanoparticles [27]. The ζ potential influences the physical stability of colloidal dispersions. High values of ζ potential confer stability to the colloidal dispersion by preventing aggregation due to electrostatic repulsion between similarly charged particles. The NLCs showed ζ potentials of -32.7 mV and -19.5 mV in the case of $NLC_{PSE10-SH}$ and $NLC_{PSE100-SH}$, respectively, while $NLC_{PSE10-OH}$ and $NLC_{PSE100-OH}$ displayed zeta potential values of -22.8 mV and -13.5 mV, respectively. The obtained ζ potential values (-20 mV/-30 mV) are considered sufficient to ensure electrostatic stabilization of the colloidal system [28]. To investigate the mucoadhesive properties of the obtained NLCs, they were loaded with 0.1% (w/w) LFR. As the log P value of LFR is 17.6 [29], the dye is highly lipophilic and insoluble in water. Indeed, Trofymchuk et al. described how unencapsulated LFR tends to form large aggregates, which are easily detected by DLS measurements [30]. As measurements performed with LFR labeled NLCs did not show the formation of such large aggregates (data not shown), the dye seemed to be fully encapsulated in NLCs.

<< Insert Table 2 >>

3.4 Preparation and characterization of SEDDS

In the final formulation of SEDDS, eugenol (log P = 2.2), benzyl alcohol (log P = 1.1), and ethanol (log P = -0.31) were used as co-solvents, polyethoxylated-35 castor oil and polyoxyethylene stearyl ether were used as surfactants, and oleyl alcohol was used as a lipid component. Water-soluble organic solvents, such as benzyl alcohol and ethanol, were selected since they could increase the solubility of drugs within the SEDDS. As the presence of a less hydrophilic co-solvent in the composition may prevent precipitation of drugs and improve the stability of the system *in vivo* [29], eugenol was added to the preparation. The obtained formulations were stable when stored at room temperature. Subsequently, SEDDS were analyzed in terms of particle size, PDI, and ζ potential. Results are listed in Table 3. Droplet size is an important parameter for emulsion stability [31]. The addition of unthiolated and thiolated PSE_{10} resulted in SEDDS of smaller size than that of SEDDS prepared with the PSE_{100}. Thiolation of PSE caused a slight increase in particle size, which was more pronounced for the short chain PSE_{10}. This observation might be explained by disulfide bond

formations. All size values, however, were below 250 nm likely guaranteeing high mucus permeating properties. Polydispersity indicates the uniformity of the formulation and showed that the droplets were uniformly distributed. The PDI values were found in a range of 0.228-0.304 in all preparations indicating a homogeneous particle distribution. The ζ potential represents an essential parameter, since the stability of a SEDDS system is directly related to the surface charge [32]. SEDDS showed a ζ potential of -9 and -17 mV in the case of $SEDDS_{PSE10\text{-}SH}$ and $SEDDS_{PSE100\text{-}SH}$, respectively (Table 3). The value obtained is in both cases unchanged from the ζ potential value from the $SEDDS_{PSE10\text{-}OH}$ and $SEDDS_{PSE100\text{-}OH}$. Negatively charged droplets should repell each other resisting aggregation and providing stability of the nanoemulsion [33].

<< Insert Table 3 >>

3.5 Mucodiffusion studies

Mucodiffusion studies were performed via the rotating tube method using tubes filled with porcine small intestinal mucus. By this assay the different physical-chemical characteristics of NLCs and SEDDS can be assessed, which influence the ability of formulations to diffuse into mucus. As illustrated in Fig. 4, unthiolated NLCs showed significantly higher diffusion properties into the first two segments of mucus than thiolated NLCs. The thiolated surfactant on the surface of NLCs is able to form covalent bonds with the cysteines of the mucus, thus hindering the diffusion of NLCs throughout mucus. The results are in agreement with data obtained recently, showing less diffusivity of thiolated compared with unthiolated nanocarriers [14]. The thiolated and unthiolated particles prepared with PSE_{10} could diffuse into deeper segments compared to the NLCs with PSE_{100}. Reason for that might be the shorter chain length of PSE_{10} lowering the possible interaction points with the mucus [34]. In comparison, PSE_{100}, which is 10 times longer, forms a PEG brush on the surface of the nanocarrier, which can interact more strongly with the mucus due to the long PEG chain. Higher interactions result in greater retention in the mucus and lower diffusivity. The results obtained by unthiolated and thiolated SEDDS are displayed in Fig. 5. Thiolated SEDDS showed higher diffusivity than unthiolated SEDDS, which is in contrast to the diffusion behavior determined for NLCs. Higher diffusivity of thiolated SEDDS has already been described in the literature previously [35]. In contrast to semi-solid NLCs, SEDDS are only based on liquid lipids containing higher amounts of surfactant to provide stability [36,37].

<< Insert Fig. 4 >>

<< Insert Fig. 5 >>

3.6 Rheological investigations

Interactions between mucus and nanocarriers were evaluated by rheological measurements. NLCs and SEDDS, showing strong interactions with mucus, cause high viscosity [14]. The results of the rheological investigations are displayed in Fig. 6 and Fig. 7. The dynamic viscosity (η^*) determined by the frequency sweep and the loss tangent ($\tan \delta$) are in good agreement, whereby the differences between the nanocarriers were more pronounced in the viscosity results. $NLC_{PSE10\text{-}SH}$ showed the highest viscosity, which was 7.62-fold higher than that of the unthiolated $NLC_{PSE10\text{-}OH}$. NLCs containing the unthiolated and thiolated short PEG chain surfactants caused a significant increase in viscosity, assuming higher interaction with the mucus by shorter PEG chain length of PSE. The $SEDDS_{PSE10\text{-}SH}$ resulted in a similar increase in viscosity like the $NLC_{PSE10\text{-}SH}$, which was 4.53-fold higher than that of the unthiolated $SEDDS_{PSE10\text{-}OH}$. In comparison to the $NLC_{PSE100\text{-}SH}$, the thiolation of $SEDDS_{PSE100\text{-}SH}$ led to more pronounced changes of results. The $SEDDS_{PSE100\text{-}SH}$ caused a 2.32-fold higher viscosity and a significant lower loss tangent compared to the $SEDDS_{PSE100\text{-}OH}$. By the strain sweep measurements shown in Fig. 6C and Fig. 7C the strength of interactions and the kind of bonding can be investigated [38]. At higher shear rates, physical interactions can be broken, while strong covalent bonds remain intact and lead to sustained high viscosity. Because of the presence of PSE_{10}-SH and PSE_{100}-SH, the thiolated nanocarriers showed significantly higher dynamic viscosity values at higher shear rates than the unthiolated ones. A reason for this observation might be the formation of disulfide bonds between the nanocarriers and cysteine moieties of mucus. Since the $SEDDS_{PSE10\text{-}OH}$ caused a significant increased viscosity compared to the $SEDDS_{PSE100\text{-}OH}$, more non-ionic interactions can be assumed. The short PEG chains are fully unfolded likely allowing each hydroxyl group to interact with mucus. On the other hand, the long PEG chains of the $SEDDS_{PSE100\text{-}OH}$ form a dense, coiled surface resulting in fewer contact points with the mucus and less interactions [39].

<< Insert Fig. 6 >>

<< Insert Fig. 7 >>

3.7 *In vitro* evaluation of mucoadhesive properties

Mucoadhesive properties were investigated on porcine intestinal mucosa via the wash-off method. As shown in Fig. 8, the amount of attached nanocarriers tends to remain constant until the end of the experiment set at 60 minutes. $NLC_{PSE10\text{-}SH}$ and $NLC_{PSE100\text{-}SH}$ exhibited 3.67- and 2.50-fold improved mucoadhesive properties compared to the corresponding $NLC_{PSE10\text{-}OH}$ and $NLC_{PSE100\text{-}OH}$, after 60 min of washing (Fig. 8A). $SEDDS_{PSE10\text{-}SH}$ and $SEDDS_{PSE100\text{-}SH}$ showed 1.70- and 1.59-fold improvement in mucoadhesive properties compared to the corresponding $SEDDS_{PSE10\text{-}OH}$ and $SEDDS_{PSE100\text{-}OH}$ under the same conditions (Fig. 8B). This increase in mucoadhesion of the investigated systems can only be explained by the thiol groups in the formulation, which could form strong covalent bonds with the mucus. The interaction of thiol groups with cysteine-rich subdomains of mucin makes the PSE_{10}-SH/PSE_{100}-SH based NLCs and SEDDS more resistant to wash-off phenomena leading to an elevated concentration of LFR remaining on the mucosal membrane. In addition, a shorter PEG chain length of PSE (PSE_{10}-OH/PSE_{10}-SH) resulted in higher mucoadhesive properties than the longer PEG chain length compounds (PSE_{100}-OH/PSE_{100}-SH). This phenomenon might be attributed to the increased ability of the short PEG chains of PSE to interact with the mucosa, causing chain entanglements with mucin chains.

<< Insert Fig. 8 >>

4. CONCLUSIONS

Lipid based nanocarrier are widely used for the delivery of poorly water-soluble drugs. However, their potential is still limited due to poor mucoadhesion. To counteract this weakness, thiolated nanocarriers with enhanced mucoadhesive properties were developed in this study. Therefore, new

thiolated derivatives of the non-ionic surfactant PSE with a short and long PEG chain with two different molecular weights, PSE_{10}-SH and PSE_{100}-SH, were synthesized and characterized. The obtained thiolated surfactants were employed for the preparation of different lipidic drug delivery systems: NLCs and SEDDS. The resulting nanocarriers exhibited a suitable size and PDI that enable mucus interaction. Mucus diffusion studies led to the following rank order with increasing diffusivity PSE_{10}-SH < PSE_{100}-SH < PSE_{10}-OH < PSE_{100}-OH for NLCs and PSE_{10}-OH < PSE_{100}-OH < PSE_{100}-SH < PSE_{10}-SH for SEDDS. Both nanocarrier systems with the thiolated surfactant showed increased mucus interaction as determined by rheological measurements and mucoadhesion studies. Specifically, the short PEG chains of PSE_{10}-SH SEDDS and NLCs showed the highest mucus interaction compared to all other formulations. The nanocarriers with the longer PEG chain of PSE_{100}-SH showed less enhancement of mucus interactions in comparison to the PSE_{10}-SH nanocarriers. Therefore, it can be assumed that the conformation of the short PEG chains is stretched and exposes the free thiol groups on the surface of nanocarriers. The long PEG chains might be in a coiled conformation, partially hiding the thiol groups and resulting in fewer interactions with the mucosa. According to the results of mucus interaction studies of SEDDS and NLCs, PSE_{10}-SH and PSE_{100}-SH are promising excipients for the development of mucoadhesive lipid-based drug delivery systems.

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