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Investigation of protein distribution in solid lipid particles and its impact on protein release using coherent anti-Stokes Raman scattering microscopy

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ABSTRACT

The aim of this study was to gain new insights into protein distribution in solid lipid microparticles (SLMs) and subsequent release mechanisms using a novel label-free chemical imaging method, coherent anti-Stokes Raman scattering (CARS) microscopy. Lysozyme-loaded SLMs were prepared using different lipids with lysozyme incorporated either as an aqueous solution or as a solid powder. Lysozyme distribution in SLMs was investigated using CARS microscopy with supportive structural analysis using electron microscopy. The release of lysozyme from SLMs was investigated in a medium simulating the conditions in the human duodenum. Both preparation method and lipid excipient affected the lysozyme distribution and release from SLMs. Lysozyme resided in a hollow core within the SLMs when incorporated as an aqueous solution. In contrast, lysozyme incorporated as a solid was embedded in clusters in the solid lipid matrix, which required full lipolysis of the entire matrix to release lysozyme completely. Therefore, SLMs with lysozyme incorporated in an aqueous solution released lysozyme much faster than with lysozyme incorporated as a solid. In conclusion, CARS microscopy was an efficient and non-destructive method for elucidating the distribution of lysozyme in SLMs. The interpretation of protein distribution and release during lipolysis enabled elucidation of protein release mechanisms. In future, CARS microscopy analysis could facilitate development of a wide range of protein–lipid matrices with tailor-made controlled release properties.

1. Introduction

Solid lipid particles have been proposed as promising drug delivery systems for peptide and protein drugs [1,2] although incorporation of sufficient amounts of drug has proven troublesome due to the hydrophobic nature of the lipids. Although direct incorporation of peptide in the lipid matrix has been shown possible using a solid–oil–water (s/o/w) emulsion method [3], protein drugs are often incorporated via an aqueous phase using a water–oil–water (w/o/w) emulsion method [4]. Hypothetically, using the w/o/w method, the drug would be expected to reside in aqueous droplets inside the melted lipid during preparation. However, the drug distribution will likely be influenced by the choice of lipid excipients, which will affect important properties of the drug delivery system, such as drug release and stability. Characterization of drug distribution in lipid drug delivery systems incorporating peptide or protein drugs has often been neglected, probably due to significant challenges associated with the analysis of drug distribution in such formulations since drug loading is often very low.

Multiple chemical imaging techniques such as infrared microscopy [5,6], Raman microscopy [7,8] and secondary ion mass spectrometry [9,10] have been utilized for determining drug distribution in solid state formulations such as solid dispersions and tablets. The techniques all suffer from different drawbacks such as comparatively poor spatial resolution for infrared microscopy, long acquisition times for Raman microscopy and some degree of sample degradation for mass spectrometry [11]. Coherent anti-Stokes Raman scattering (CARS) is a non-linear imaging technique, which, like Raman microscopy, utilizes Raman scattering, but with much faster acquisition times [12]. CARS signals are generated when a pump photon of frequency ω_p, a Stokes photon of frequency ω_s and a probe photon with frequency ω'_p interact in time and space within a sample to generate the anti-Stokes signal

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with a frequency of $\omega_{as} = \omega_p - \omega_s + \omega'_p$ [12,13]. When the frequency difference between the pump photon and the Stokes photon matches a specific molecular vibrational resonance of the sample, the CARS signal can be strongly enhanced, which allows up to video rate imaging. The probing of molecular vibrations also makes the technique potentially chemically specific without the need for exogenous labels [13]. As CARS microscopy is inherently confocal, the technique allows three-dimensional imaging by altering the focal plane of the laser beams [12,13]. CARS microscopy has found use for the imaging of living tissues [14] and cells [15] and, due to its speed, it has also been used in the imaging of dynamic situations such as release studies [11,16,17] and lipid digestion [18]. The combination of CARS microscopy with more traditional, yet complimentary, analysis methods strengthens interpretation of the results. Electron microscopy is a well-established method that can provide visualization of the structure of particulate systems at very high resolution [19]. The combination of CARS and electron microscopy allows the validation of the observed drug distribution by linking drug distribution with structural information.

The drug distribution in solid lipid particles can affect the drug release profiles [20]. The release of lysozyme from solid lipid microparticles (SLMs) has been found to depend on the lipase degradation of the lipid matrix when using triglycerides such as trimyristin and tristearin as lipid excipients. This was not the case when using a mixture of mono- (47.9%), di- (44.2%) and tri-glycerides (7.9%) where the release was found to be lipase-independent [21]. The difference in drug release behavior was explained by structural differences in the particles, with the drug distribution hypothesized to be different in the triglyceride particles compared with the particles made from the lipid mixture. Besides the influence of the preparation method, it is therefore also interesting to evaluate the effect of different lipid excipients on the drug distribution.

In the present study, lysozyme was incorporated in SLMs using a melt dispersion method. In a melt dispersion, the lipid is heated above its melting temperature and emulsified to create small droplets of melted lipid. Drug can be added during preparation and after rapid cooling, SLMs are formed. Lysozyme was used as a model hydrophilic protein to evaluate the difficulties associated with incorporation of such compounds in SLMs. The distribution of lysozyme in SLMs was investigated using CARS microscopy and structural features were confirmed using electron microscopy as a complimentary technique. To our knowledge, this is the first successful study involving the determination of protein distribution in a solid dosage form using CARS. In order to determine the difference in internal protein distribution following lysozyme incorporation either as an aqueous solution or as a solid powder, particles were prepared using both s/o/w and w/o/w methods. Different lipid excipients were used to prepare SLMs to investigate the influence of lipid type on the drug distribution and consequent drug release characteristics.

2. Materials and methods

2.1. Materials

The lipid Dynasan 114 (glyceryltrimyristate, TG14) was kindly provided by Cremer Oleo (Hamburg, Germany), and the lipids Precirol ATO 5 (Glyceryl distearate type 1, GDS) and Geleol mono- and diglycerides NF (Glycerol monostearat 40–55 type 1, GMS) were obtained from Gattefossé (Lyon, France). *Thermomyces lanuginosus* lipase A solution was a gift from Novozymes (Bagsværd, Denmark). Phosphatidyl choline (Lipoid S PC, >98%) was purchased from Lipoid (Ludwigshafen, Germany). Lysozyme from chicken egg white (70,000 U/mg, mass median diameter of 7.0 ± 0.5 μm), sodium taurocholate (>95%), polysorbate 80 and polyvinyl alcohol (average Mw 85,000–124,000, 87–89% hydrolyzed) were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of solid lipid microparticles

Lysozyme-loaded SLMs were prepared as previously described [21] based on the method described by Reithmeier et al. [3]. In short, 300 mg of lipid excipient was melted at 80 °C and mixed vigorously with 100 μL of lysozyme aqueous solution (600 mg/mL) for particle preparation by the w/o/w method (TG14(w), GDS(w) and GMS(w)) or 60 mg of lysozyme powder for preparation by the s/o/w approach (TG14(s), GDS(s) and GMS(s)) for 5 s at room temperature (RT). This blend was then mixed with 3 mL of a hot (80 °C) polyvinyl alcohol (PVA) aqueous solution (1%, w/v) for 10 s at RT, and then poured into 150 mL of cold (5 °C) PVA aqueous solution (0.1%, w/v) while stirring. The suspension was stirred for 5 min and filtered through a fine Whatman 50 (2.7 μm) filter. The SLMs were washed multiple times with a total of 60 mL of purified water and dried at RT overnight under vacuum. Lysozyme has previously been found to be stable during this preparation process [21].

2.3. Physicochemical characterization of the SLM

The sizes of the SLMs were measured using laser diffraction with a Mastersizer 2000 (Malvern, Worcestershire, UK). The samples were dispersed using a dry powder dispersion unit (Scirocco, Malvern, UK) at a feed pressure of 2.5 bar and analyzed in triplicate (3 different batches, 1 measurement each) with a reference refractive index of 1.553.

The drug entrapment efficiency (EE) and drug loading (DL) were determined by dissolving 10 mg of the SLMs in 2 mL of chloroform, followed by extraction of lysozyme with 3 mL of purified water. The mixture was rotated for 15 min and after phase separation a 500 μL sample was taken from the aqueous phase and analyzed by HPLC. EE was calculated using Eq. (1):

$$EE(\%) = m_{drug}/m_{total} * 100 \quad (1)$$

where m_{drug} is the measured mass of lysozyme in the weighed SLMs and m_{total} is the theoretical mass of lysozyme in the weighed SLMs (assuming 100% entrapment). DL was calculated using Eq. (2):

$$DL(\%) = m_{drug}/m_{particles} * 100 \quad (2)$$

where $m_{particles}$ is the mass of the weighed SLMs.

The amount of lysozyme accessible on the particle's exterior surface was quantified by incubating 15.0 mg of particles in 5 mL of a 5% (v/v) polysorbate 80 solution for 5 min under continuous rotation at 37 °C. The samples were centrifuged for 3 min at 15,000 rpm (19,000 *g* at r_{max}) and the amount of lysozyme in the supernatant was determined by HPLC.

2.4. CARS imaging

CARS spectra and images were acquired with a Leica CARS microscope (Leica TCS SP8 CARS). The instrument contains a Leica DMI 6000 inverted microscope equipped with a picoEmerald tunable light source (APE, Berlin, Germany) and two forward- and two EPI-directed PMT detectors. The Stokes beam at 1064 nm was emitted from the Nd:YVO_4 laser while a tunable pump/probe beam at 780–940 nm was generated from the second harmonic (532 nm) by an optical parametric oscillator (OPO). The pulse width of the source was 5–7 ps corresponding to the Raman line width of 2–3 cm^{-1}. The pulses from the two sources were temporally and spatially overlapped on the focal plane of the microscope. Up to 100 mW of average power from both the pump and the Stokes source was delivered to the sample. An infrared corrected 25× water immersion objective (Leica HCX IR APO L 25×/0.95 W) was used. The CARS signal from the sample passed through a broadband filter block (560–750 nm) and was detected in the EPI-