Journal of Controlled Release 147 (2010) 202–210

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

The mechanism of protein release from triglyceride microspheres

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ARTICLE INFO

Article history:
Received 23 March 2010
Accepted 17 July 2010
Available online 24 July 2010

Keywords:
Lipid microspheres
Confocal microscopy
Particle distribution
Protein release
Release mechanism

ABSTRACT

The purpose of this study was to reveal factors that have an impact on the protein release kinetics from triglyceride microspheres prepared by spray congealing. We investigated the effect of protein particle size, morphology and distribution on protein release from microspheres by confocal laser scanning microscopy (CLSM). The microspheres were loaded with three types of model particles made of FITC-labeled bovine serum albumin: freeze dried protein, spherical particles obtained by precipitation in the presence of PEG and micronized material. Investigation by light microscopy and laser light diffraction revealed that the freeze dried material consisted mainly of app. 29 μm elongated shaped particles. The precipitated BSA consisted mainly of 9.0 μm diameter spherically shaped particles while the micronized protein prepared by jet milling consisted of 4.9 μm sized rounded particles of high uniformity. Microspheres were embedded into a cold-curing resin and cut with a microtome. Subsequent investigation by CLSM revealed major differences of distribution of the polydisperse protein particles inside the microsphere sections depending on the type of BSA that was used. Particles of micronized and precipitated protein were distributed almost throughout the microsphere cross section. The protein distribution had a marked impact on the release kinetics in phosphate buffer. Large protein particles led to a considerably faster release than small ones. By staining the release medium we demonstrated that in all three cases there was a strong correlation between protein release and buffer intrusion.

1. Introduction

Protein and peptide delivery applications are an area of growing interest in the field of controlled release technology due to an increasing number of potential therapeutic agents made available by the progress in biotechnology and genetic engineering [1]. Triglycerides have received attention as protein and peptide drug delivery systems due to their favorable properties as physiological substances of high biocompatibility [2–4]. This qualifies them as a promising alternative to commonly used polymeric matrix materials such as poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(D,L-lactide-co-glycolide) (PLA) [5–9]. The incorporation of drugs into lipid microparticles or monolithic matrices and controlled release thereof has already been reported for several peptide, protein drugs and model substances such as insulin [10–12], somatostatin [13], interferon α-2a [14], BSA, hyaluronidase [15], lysozyme, brain derived neurotrophic factor [3], interleukin-18 [16] and thymocartin [17]. Confocal microscopy studies devoted to analyzing drug release mechanisms for monolithic triglyceride cylinders [18] revealed that the release was diffusion controlled and triggered by the intrusion of water into the lipid matrix. The process depended strongly on the wettability of the material [19]. This in vitro release was neither accompanied by erosion nor by swelling phenomena [20]. In contrast, information and data on release mechanisms for lipid microspheres are still sparse. Release studies (usually conducted with microspheres) take into account factors such as apparent diffusivity, drug solubility, drug loading, microsphere size and nature of lipid and attempt to draw conclusions on release mechanism [21–25]. However, despite a decade of research on protein incorporation into lipid microparticles, there is still no data available on their microstructure and its impact on release mechanisms and kinetics [26].

The aim of this study was to close this gap by cross sectioning resin embedded protein loaded triglyceride microspheres using a microtome. Our goal was to correlate the microstructure with protein release. We also investigated the protein particle size, morphology and distribution in the microspheres. To this end we loaded triglyceride microspheres with three types of model protein particles made of FITC-labeled bovine serum albumin (FITC-BSA) and counter-stained pores inside the microspheres using sulforhodamine 101 (SRH) as a dye that was added to the release buffer. This allowed us to follow the fate of the proteins in microsphere sections using confocal laser scanning microscopy (CLSM). Overall we intended to see if there was a correlation between classical in vitro release data and the fluorescence intensities of microsphere cross sections.

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doi:10.1016/j.jconrel.2010.07.110

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, Cohn fraction V, Mw 66,000 Da), bicinchoninic acid disodium salt (BCA), sulforhodamine 101 hydrate (SRH, Mw 607 Da) and Sephadex G25 were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany). Fluorescein isothiocyanate was obtained from Fluka (Buchs, Switzerland). Polyethylene glycol (PEG) with a molecular weight of 6 kDa, dichloromethane (DCM) and dimethylsulfoxide (DMSO) were obtained from Squarix (Marl, Germany). Copper-(II)-sulfate pentahydrate and sodium azide were purchased from Merck (Darmstadt, Germany). Hydrogenated palm oil (S-154, Softisan® 154,) was a gift from Sasol (Witten, Germany). It consists of a triglyceride mixture of natural, hydrated, evenly chained and unbranched fatty acids, consisting of app. 97% palmitic and stearic acid, with a melting point of 53–58 °C. Technovit 7100 was purchased from Heraeus Kulzer GmbH (Germany). P. a. grade chloroform was purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS) was obtained from Gibco (Gibco/Invitrogen, Karlsruhe, Germany) and cell culture medium (Leibovitz) Tween® 80 was obtained from Sakura Finetek Europe (Zoeterwoude, Netherlands).

2.2. Preparation of different types of FITC-BSA particles

2.2.1. FITC labeling of BSA and production of freeze dried FITC-BSA particles (F-FITC-BSA)

100 mg of FITC was dissolved in 8 ml DMSO and slowly added to 1 g BSA in 100 ml 0.1 M disodium carbonate buffer pH 9.0. The mixture was gently stirred in the dark at room temperature for 2 h. Low molecular weight compounds and excess FITC were removed by gel filtration with sephadex G-25 column (2.5 × 40 cm). We checked for free dye after ultrafiltration (MWCO 30 kDa) to make sure that the protein was free of unreacted FITC. Fractions containing labeled protein were freeze dried at 6 °C and 0.12 mbar using a benchtop freeze-dryer (Beta 2–16 with LMC-2 system control, Christ, Osterode, Germany). For complete drying the temperature was increased to 20 °C for additional 6 h. The vacuum was removed by filling the chamber with dry nitrogen. The F-FITC-BSA was ground in a mortar inside a glovebox flooded with dry nitrogen and stored at −20 °C in a desiccator until further use.

2.2.2. Preparation of FITC-BSA particles by precipitation (P-FITC-BSA)

FITC-BSA was co-lyophilized with PEG to obtain precipitated protein particles according to a modified method described in [27]. In brief: equal amounts of BSA and PEG (1:1 mass ratio) were dissolved in water and freeze dried as described previously. The particles were washed immediately with 50 ml of methylene chloride to dissolve PEG under sonication. After centrifugation at 15.974 g for 5 min, the supernatant was decanted. This procedure was repeated 3 times to remove PEG quantitatively. The product was then frozen in liquid nitrogen. By applying a vacuum, the organic solvent was removed to yield the final precipitated FITC-BSA (P-FITC-BSA) which was stored at −20 °C until further use.

2.2.3. Preparation of micronized FITC-BSA particles (M-FITC-BSA)

For the preparation of micronized BSA (M-FITC-BSA), F-FITC-BSA (see Section 2.2.1) served as raw material. The material was processed using a MC One® jet mill (Jetpharma, Balerna, Switzerland). For an individual cycle 500 mg of protein was micronized. The milling pressure was 10 bar and the feeding pressure was 1 bar above to prevent blow back of the powder. The feeding rate was kept constant at 120 mg/min. In order to exclude any humidity from the protein, the whole jet mill was operated in a glovebox filled with dry nitrogen. A hygrometer was installed within the glovebox to check the air humidity permanently (relative humidity below 2%). The obtained micronized FITC-BSA protein (M-FITC-BSA) was stored at −20 °C in a desiccator until further use. The moisture content of different FITC-BSA particles was determined by Karl Fischer titration (AquaStar C2000, EM Science, Cherry Hill, NJ).

2.3. Preparation of protein loaded lipid microparticles by spray congealing

For the preparation of particles in batch sizes of 4 g, a modified spray dryer was used. The spray congealing apparatus was composed of a nozzle in a heating jacket and the conventional glassware of a spray dryer (Mini-Spray Dryer B290, Büchi Labortechnik, and Flawil, Switzerland). The stream of air was set such that after an initial phase of approximately 30 min the temperature in the spraying tower and the cyclone had reached approximately −10 °C. FITC-BSA 4% was suspended in the molten lipid (softisan-154) at 70 °C with an ultraturrax (TP 18/10 equipped with S 25 N 10 G dispersing tool; IKA Laboratory Technology, Staufen, Germany) for 2 min at 10.000 rpm in the reservoir container of the nozzle [11]. The FITC-BSA suspensions were then atomized at 70 °C with 4 bar spraying pressure and 0.5 bar atomizing air pressure. The resulting particles were collected and stored at −20 °C until further analysis. The product yield was calculated as the mass of microspheres obtained in the production vessels in relation to the mass of the suspension introduced in the spraying nozzle.

2.4. Investigation of particle size distribution and morphology of protein particles and lipid microspheres

Particle size distributions were determined by laser light diffraction (Mastersizer 2000, Hydro 2000 μP dispersion unit; Herrenberg, Germany). FITC-BSA particles were suspended in 18 ml degassed isobutanol (refractive index 1.39). For disaggregation of agglomerates ultrasound was applied (48 kHz, 20 W) for 10 s prior to measurement under constant stirring at 2000 rpm. Each sample was measured 5 times for 20 s. As characteristic values representative of particle sizes $d_{0.5}$ and $d_{0.9}$ values (percentage of particles that are smaller than the given value) were assessed. Protein particle morphologies were investigated by scanning electron microscopy (SEM). The particles were mounted on aluminum stubs using conductive carbon tape (LeitTabs; Plannet GmbH, Germany) and coated with gold by sputtering three times for 20 s (SEM Autocoating unit E2500; Polaron equipment LTD, UK). Protein loaded lipid microspheres were suspended in 2 ml of an ethanol–water mixture (68.2% V/V) on a vortex mixer (Vortex-Genie; Scientific Industries, New York, USA) for 10 s. The sample was added to the dispersion unit filled with the dispersion medium under constant stirring at 2500 rpm. The particle sizes were again recorded as $d_{0.5,}$ $d_{0.9}$ and mean diameter. Protein loaded lipid microsphere morphology was investigated by SEM as described previously.

2.5. Thermal behavior of lipid microspheres

Differential scanning calorimetry (DSC) was used to determine the crystal structure of lipid matrices. Samples of 3.5 mg ± 0.05 mg of the bulk lipid or microspheres without protein were sealed into AutoDSC aluminum sample pans (TA Instruments, Alzenau, Germany) and DSC thermograms were recorded using a 2920 differential scanning calorimeter (TA Instruments, Alzenau, Germany) with an empty pan as reference. Scans were recorded between −20 °C and 90 °C with a rate of 5 K/min after equilibration at −20 °C for 5 min [11]. The obtained data were evaluated with the DSC-software Universal Analysis 2000 for Windows 98/NT, version 2.5 H (TA instruments, Alzenau, Germany). To investigate the influence of incubation time with water and of release conditions on the morphology of protein