Preparation of HSA containing PLGA particles by a novel emulsifier

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Introduction

Poly(lactic-co-glycolic acid) (PLGA) is one of the most promising FDA-approved encapsulating compounds because of its good biodegradability, biocompatibility and low toxicity. As more and more new protein drugs are invented, there is an increasing demand to find suitable methods and conditions to prepare composites, whose properties (e.g. particle size distribution, encapsulation efficiency, release profile) fulfil the strict requirements of drug formulation.
The drug incorporation capability, the size and the release characteristics of the drug-loaded particles determine their applicability; nevertheless, these properties can be mostly influenced by the quality and quantity of the used surfactant stabilizer. Polyvinyl alcohol was found to be very effective emulsifying agent both in its encapsulating property and its size decreasing (by stabilizing nanosized emulsion droplets) effect. However, certain amount of residual PVA associated with the surfaces of the particles can influence the polymer degradation as well as it can inhibit the protein release. Poloxamer is also a useful emulsifier to produce nanoparticles encapsulating drug proteins in an efficient manner. Although new stabilizers could possess some advantageous properties which does not characterize the previously mentioned emulsifiers.
The aim of this study was to investigate the conditions and parameters of the preparation of PLGA-HSA composite particles using a novel surface active agent. BASF has recently lauched new ranges of readily biodegradable non-ionic surfactants namely Lutensol XL types. These are short-chain alcohol ethoxylates with a highly dynamic wetting action, their high emulsifying power is comparable to the surfactants based on longer-chain alcohols.

Materials and methods

The PLGA polymer used was Resomer® 502 H with a molecular weight of 8000 Da (Boehringer Ingelheim, Germany). HSA in phosphate buffered saline (pH=7.4) was obtained from Trigon Biotechnological Ltd. (Hungary). Poly(vinyl alcohol) M_w=30000-70000 and phosphate buffered saline tablets were from Sigma. Lutensol XL80 (an alkyl polyethylene glycol ether made from a C10-Guerbet alcohol and eight ethylene oxides) was from BASF. Poloxamer (M_w=8 350, BASF, Ludwigshafen, Germany, Lutrol® F68) was a kind gift from Pharmaceutical Technology Department, University of Szeged, Hungary. PVP (M_w=350 000) was obtained from Serva, Heidelberg, Germany. Dichloromethane was supplied by Spektrum-3D, Hungary. The micro BCA protein assay reagents were purchased from Pierce, Bonn, Germany.
The nano- and microspheres containing HSA as a model protein were prepared by double emulsion–solvent evaporation method (Feczkó et al, 2007). Formed nano- and microparticles were isolated by ultracentrifugation at 50000g and 20000g, respectively (Beckman Optima Max-E) for 25 min. The particles were washed with distilled water to remove the residual surfactant and then lyophilized (Leybold-Heraeus Lyovac GT2).
To determine the encapsulation efficiency of the prepared particles, both the encapsulated protein and the remaining fraction in the supernatant were analyzed. Briefly, 20 mg of the particles was dissolved in 3 ml of 1 N NaOH. Unloaded PLGA particles were also prepared and hydrolysed under the same conditions to which different quantities of protein were added to obtain calibration curve. The protein content was estimated by a Biuret method relying on the reduction of Cu^{2+} by proteins in alkaline solution. 1 ml of Biuret reagent (30 mM KI, 100 mM K-Na-tartarate, 30 mM $CuSO_4$, 3.8

M NaOH) was added to each 3 ml extract and the violet colour was quantified spectrophotometrically [12] at 546 nm (Pharmacia LKB, Biochrom 4060) after 15 min at room temperature. The amount of protein, that was not encapsulated, was measured in the supernatant by the micro BCA (bicinchoninic acid) protein-assay (Pierce Biotechnology, Inc.).
Size of the particles was determined by laser scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) at 20 °C. The average particle size was expressed in volume mean diameter. Morphology of the particles was monitored by environmental scanning electron microscopy (Philips XL-30 ESEM). Samples were prepared for analysis with the following method: centrifuged and lyophilized particles redispersed in distilled water were dropped onto grid and dried under room temperature, and were vacuum-coated for 3 min with a mixture of gold and palladium.
The protein release kinetics of particles was examined in a short interval. 40 mg of lyophilized particle was incubated for five days in 10 ml phosphate-buffered saline (pH 7.4) containing 0.03 % sodium azide as bacteriostatic agent in an Eppendorf tube. The temperature of air-bath incubator was maintained at 37 ºC with continuous agitation at 170 rpm. At each sampling time, 1 ml of each sample was ultracentrifuged at 50000 g, and the clear supernatant of the release medium was withdrawn and replaced with fresh medium. The released protein was investigated by micro BCA protein assay.

Results and discussion

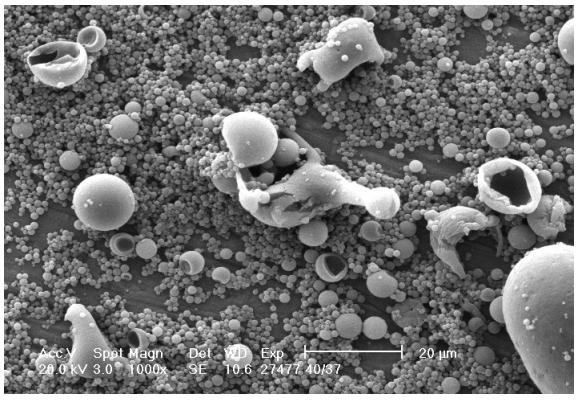
Primarily, preparation of nanosized particles was targeted. As Lutensol XL 80 was not studied before for this purpose, the results of other emulsifiers should have been considered for the experimental design. The HSA loading of the particles was aimed to be smaller than 10 % w/w with respect to the PLGA concentration, as we found that recently with the frequently used PVA emulsifier (Feczkó et al., 2007), the entrapment could be maximized (>90 %) in this range by applying suitable surfactant concentration. The PLGA concentration was kept constant at 1 % w/v relative to the external water phase volume. Applying 4 % of Lutensol XL 80 resulted in the maximum encapsulation efficiency, 62 %. During synthesis of microparticles, higher entrapment efficiency was obtained, 70.0 % at 2 % emulsifier concentration.
The combination of emulsifiers might elevate the encapsulation efficiency even for nanoparticles (Vandervoort et al., 2002). Since with 1 % w/v of polyvinyl alcohol alone, nanoparticles (average diameter: 0.15 μm) could be prepared with an excellent encapsulation efficiency (>90 %) (Feczkó et al., 2007), it seemed to be a suitable emulsifier which could be combined with Lutensol XL 80. Although adding 1 % w/v PVA to 2 % w/v Lutensol XL 80 solution, the encapsulation efficiency exceeded 90 %, but unfortunately, other favourable features of the particles were lost (see later). It is possible to produce particles with an incorporation efficiency higher than 90 % by 1 % w/v PVP alone, but nanoparticles were formed only in a small ratio. Combination of 2 % w/v Lutensol XL 80 and 1% w/v PVP slightly enhanced the encapsulation (65 %). By producing microparticles with the same composition of surfactants, 75 % of the initial HSA was encapsulated. Most polymers such as PVP are not able to stabilize the emulsion as strongly as PVA does. Poloxamer is one exception, which can be considered as a valuable alternative for PVA. We produced particles with an average diameter of 0.45 μm using 2 % w/v poloxamer (Lutrol F68), with an entrapment efficiency of 70 % (Feczkó et al., 2007). Upon combining 2 % w/v Lutensol XL 80 with 2 % w/v poloxamer, the encapsulation efficiency was 76 % during particle preparation with size around 1 μm. The same emulsifier concentration ratio provided 90 % of incorporation efficacy upon formulating large microparticles.
The amount of stabilizer added has an effect also on the nanoparticle size. Most importantly, if the stabilizer concentration is too low, aggregation of the polymer droplets will occur and little if any nanoparticles will be recovered. Too much emulsifier can diminish the encapsulation efficiency (Hans et al., 2002). Beside its good emulsifier properties, PVA possessed an undesirable effect, namely it caused strong irreversible agglomeration of the nanoparticles after centrifugation. This is probably due to the insufficiently high zeta potential in absolute value, which is followed by the removal of the most of the surface active agent. The aggregation should be avoided during this

process because it may detrimentally influence the application possibility of the particles. Wolf et al. (2003) suggest substituting poloxamer for PVA in order to avoid agglomeration after lyophilisation. Further, poloxamer has been shown to reduce capture by macrophages and increase the time for systemic circulation. However, in our investigations poloxamer was as efficient as PVA in encapsulation only if it was used at a very high concentration. Moreover, low concentration of poloxamer would be necessary for more negative zeta potential. In fact, we realized that poloxamer could not completely inhibit the adhering of the particles after centrifugation. However, Lutensol XL 80 was suitable for preparing particles with a diameter around 1 μm, which did not show aggregation. It must be emphasized again that this emulsifier was not as efficient in encapsulation efficiency and size decreasing as PVA or poloxamer. With this latter two surface active agent, submicron sized particles could be formed easily, while only about 50 % of the particles produced by Lutensol XL 80 was smaller than 1 μm.

The influence of the combination of emulsifiers on the particle size was examined. Significant differences were observed during the particle formation with size of about 1 μm. Adding PVA to the Lutensol XL 80 stabilizing solution, most of the particles were in the submicron range (average diameter 0.4 μm), but the particles aggregated after centrifugation. Giving PVP or Lutrol F68 surfactant to the Lutensol XL 80 resulted in particles that were easy to redisperse. However, with PVP, the polydispersity of the particles was high. On the other hand, combination of Lutensol XL 80 and Lutrol F68 provided particles with similar size distribution to that formed by Lutensol XL 80 alone. Particles were easily redispersable only if the Lutensol XL 80 concentration was not exceeded by the Lutrol F68 concentration. Interestingly, the size of large microparticles did not change significantly due to applying additive emulsifiers beside Lutensol XL 80. Typical mean diameter was between 260 and 300 μm with 90 % of the particles in the range of 100-600 μm.

The structure of particles was investigated by an environmental scanning electron microscope. Figures 1 and 2 show the particles formulated by various emulsifier compositions. It can be concluded from the images that particles prepared by Lutensol XL 80 + Lutrol F68 (Fig. 2A) were most homogenous and their shape was mostly spherical. Most of the small particles formulated by Lutensol XL 80 alone also had spherical shape (Fig. 1A). Nevertheless, there can be seen some huge hollow particles which can be characterized by large holes surrounded by a thin shell. These particles did not weigh much in the total mass, and they could be easily removed. Particles produced by Lutensol XL 80 + PVP showed rather polydisperse distribution, and their shape was often irregular (Fig. 2B). The inhomogeneous distribution and irregular shape can be attributed to the weaker stabilizing effect of the emulsifier. The structure of big microparticles prepared by Lutensol XL 80 or its combination with the mentioned emulsifiers did not show much variation, that is why, only the ones formed by Lutensol XL 80 are represented (Fig. 1B). They all had porous surface as well as partly spherical and irregular shape.

A



B

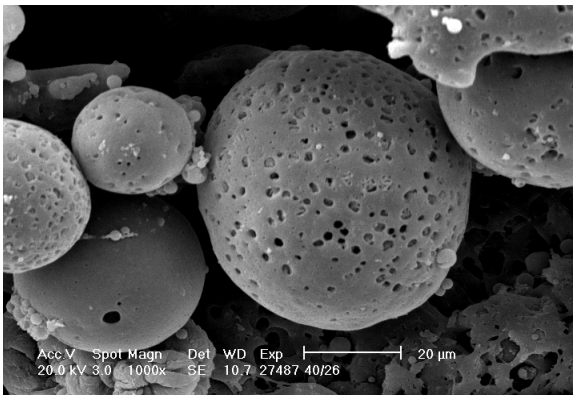


Figure 1: SEM images of particles (A-small, B-big) prepared by Lutensol XL 80.

A B

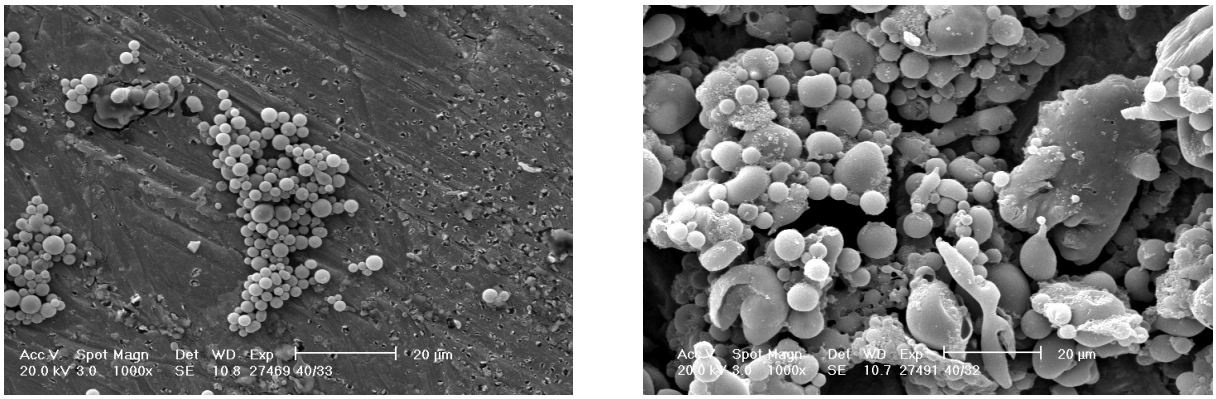


Figure 2: SEM images of particles formulated by Lutensol XL 80 + in combination with Lutrol F68 (A) and with PVP (B).

HSA release was studied during a short period of 5 days. The release profile of particles prepared by Lutensol XL80 was very similar to that of particles formulated by any combination of the emulsifiers. The initial burst was between 20 % and 25 %, which was followed by a continuous slow release reaching 28-37 % after 5 days. The release of particles differed significantly only in the case of large microparticles formed by Lutensol XL80 + PVP. After an initial burst of 10 % the released protein was only cca. 18 % after the studied period.

4. Conclusion

PLGA-HSA composite particles were prepared by a novel readily biodegradable emulsifier, Lutensol XL 80, in order to improve controlled drug delivery systems. Particles with mean size of around 1 μm as well as large microparticles could be formulated. Lutensol XL 80 was found to be a suitable emulsifier in the double emulsion method, although its emulsifying ability was weaker than that of PVA or poloxamer. The relatively low encapsulation efficiency could be increased by combining the conventional emulsifiers with the new surfactant. The main benefit of the Lutensol XL 80 is the excellent redispersability of the formed particles after centrifugation. This property could be saved when the Lutensol XL 80 was used in combination with PVP or poloxamer in suitable ratios. The protein release kinetics of particles generally did not change when the emulsifiers were combined, though, further investigations are obviously necessary in this respect. The short release studies implied slow delivery of protein in all of the investigations.

References

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