Application of lipospheres in cell biology and tissue engineering

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INTRODUCTION

Lipospheres (LS) consist of solid microparticles with a mean diameter usually comprised between 0.2 and 500 μm, composed of a solid hydrophobic fat matrix, where the bioactive compound(s) is dissolved or dispersed (Cortesi R. et al. 2002).
The choice has fallen on such formulations because LS, like emulsion and liposomes, are physiologically well-tolerated ingredients, already been approved for pharmaceutical use in humans; in addition, similar to polymeric particles, their solid matrix can protect drugs against chemical degradation and allow modulation of drug release profile. Thus lipid microspheres combine the advantages of polymeric particles, fat emulsion and liposomes, avoiding some of their typical disadvantages, such as cytotoxic effects after phagocytosis, toxic effects of organic residues after the production of polymers and lack of large industrial-scale production.
Lipid based microparticles have been successfully proposed for the delivery of a variety of conventional drugs including: antibiotics, antiinflammatory compounds, vaccines and adjuvant. Summarizing, the paper describes the optimization, by factorial design, of the preparation procedure for lipospheres.

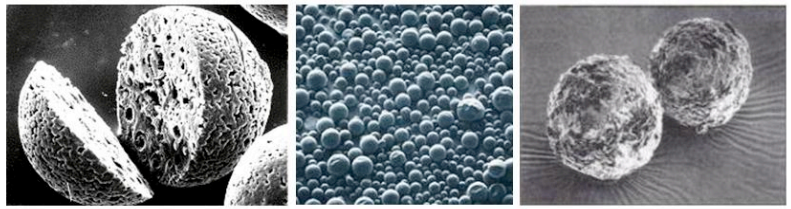

Fig 1. Representative SEM photomicrographs of lipospheres prepared with glyceryl tripalmitate and glyceryl monostearate.

MATERIALS AND METHODS

Preparation of LS by melt dispersion technique : The different lipid mixtures (triglyceride/monoglyceryde % 90/10 to 70/30) were melted at 70-75°C (depending on their melting point) and then emulsified into 15 ml of an external aqueous phase containing 0.5-2% (w/v) of PVA as the dispersing agent. The emulsion was stirred at 8000 rpm using an IKA T25 Ultra-turrax (IKA Labortechnik, Germany) for 2 min. After stopping the agitation, the milky emulsion was rapidly cooled to about 10°C by immersion in a cool water bath. The obtained lipospheres were isolated by centrifugation (10 min at 5200 g) using a Sigma 2-16 centrifuge (Sigma, Germany); the obtained pellet was washed 3 times in pure water and finally dried under vacuum. For the preparation of

cationic lipospheres, to the neutral 5 lipid mixture, different cationic surfactants were added: $DDAC_{18}$, $DDAB_{16}$, $DDAB_{12}$ and $C_{18}C_{18}VBr_2$.

Experimental design and statistical data : For investigating the effect and the influence of different experimental parameters on the dimensional and morphological characteristics of lipospheres, a randomized central composite face-centered design (CCF) consisting of 17 runs, was performed. In order to obtain important information about the replicability of the experiment, the estimation of the lack of fit of the model, and the detection of curvature in the experimental domain, three of the 17 runs were represented by three center point. The varying parameters were the quantity of glyceryl monostearate respect to glyceryl tripalmitate, PVA concentration and the oil to water ratio quantity. The experimental design and the evaluation of the experiments were performed by the PC software MODDE 8.0 (Umetrics AB, Umeå, Sweden), followed by multiple linear regression (MLR) algorithms.

Lipospheres dimensional and morphological analysis : The morphology of lipospheres was evaluated by optical-stereo microscopy (Nikon SMZ 1500 stereo microscope, Tokio, Japan) and their size and size distribution (by number) were determined by photomicrograph analyses (EclipseNet version 1.16.5, Laboratory Imaging s.r.o. for Nikon B.V.). For every preparation a sample of 500-1000 beads was analyzed, in order to calculate the mean diameter and standard deviation.

RESULTS AND DISCUSSION

Preparation of tripalmitin based lipospheres for lab-on-a-chip applications: general consideration : With the aim to develop lipospheres for possible application in cell biology and tissue engineering, the lipospheres composition and the experimental set-up were firstly investigated, by an intuitive approach. In the second part of the study a number of selected process parameters were examined in a deeper statistical fashion by an experimental design approach.
An intuitive approach, changing-one-separate-factor-a-time (COST) was initially employed. This strategy was performed to determine the lipid composition that would lead to the best microparticles in term of morphology, surface and dimensional characteristics. The investigated parameters were: (a) triglyceride type, (b) presence of dye and (c) type of partially hydrophilic component.

COST approach: effect of triglyceride on lipospheres characteristics : Among the four different triglycerides tested (glyceryl trimyristate, glyceryl tripalmitate, glyceryl tristearate and glyceryl tribehenate), the use of glyceryl tripalmitate led to lipospheres with the best morphological features, such as: regular spherical shape, smooth surface and narrow cumulative undersize distribution (%), with a mean diameter of 11.7 ± 2.7 μm (Fig. 2, left). In addition, and very importantly, tripalmitin based lipospheres were more easy to isolate in a dry, free flowing form by a simple procedure based on centrifugation. For these reasons glyceryl tripalmitate was selected as main lipophilic component and it was employed for all other liposphere preparations.

Production of colored lipospheres : Different dyes were included in the liposphere formulation to obtain microparticles of different colours, in order to (a) improve their visibility under optical microscope during cell biology experiments. To this aim, a number of lipophilic dyes were tested for inclusion into lipospheres. It was possible to produce and isolate microparticles with optimal morphological characteristics and a homogeneous distribution of the dye. Since the presence of the

dye had no effects on the general characteristics of lipospheres, further experiments were conducted always including a dye in the formulation.

Effect of polar component on lipospheres characteristics : It has been previously demonstrated that it was impossible to produce morphologically acceptable LS without the presence of at least 1% of a partially hydrophilic polar compound such as glyceryl monostearate. In the present paper we compare the effect of different polar compounds on liposphere characteristics, namely: glyceryl monostearate (GMS), satured polyglycolised glycerides SuppocireBP® (SBP), SuppocireNA15® (SNA), Gelot64® constituted of glyceryl stearate and of PEG-75 stearate (G64), rac-1-oleoyl glycerol (OG), stearoyl macrogol glycerides Gelucire50/13® (G51), semi-synthetic glycerides SuppocireDM® (SDM), 2-octyldodecyl myristate (MOD), isostearyl isostearate (ISI).
All formulations were satisfactory in terms of mean diameter, except those including Gelot64® (G64) and Gelucire50/13® (G51) that were rather small, (mean diameter 3.77 ± 2.67 μm and 2.63 ± 2.87 μm, respectively). By a detailed analysis, including the study of the polydispersity, shape, surface, dye encapsulation and presence of aggregates, it can be concluded that the lipid composition leading to the best LS was a mixture glycerol tripalmitate/glycerol monostearate.

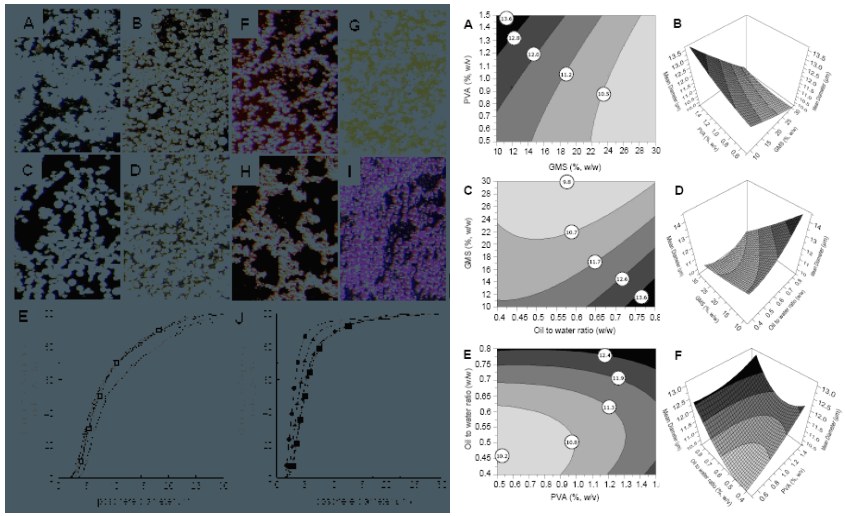

Fig. 2. Left: effect of lipid composition (type of triglyceride) (panels A-E) and dye (panels F-J) on lipospheres characteristics. Stereo microphotographs of lipospheres prepared with glyceryl trimyristate, LS1-w (A); glyceryl tripalmitate, LS2-w (B); glyceryl tristearate, LS3-w (C) and glyceryl tribehenate, LS4-w (D). Bars correspond to 40 μm. Stereo microphotographs of stained lipospheres prepared with sudan black, LS2-b1 (F); oil red O, LS2-r1 (G); scarlet red, LS2-r2 (H) and fat red bluish, LS2-r3 (I). Bars correspond to 40 μm. In panels E and J are reported the cumulative frequency distribution plots of following lipospheres batches: LS1-w ( ), LS2-w ( ), LS3-w (□), LS4-w ( ), LS2-b1 ( ), LS2-r1 ( ), LS2-r2 (■) and LS2-r3 (●).Right: response surface plots from the 3-level factorial design showing the effect on liposphere mean diameter as a function of GMS quantity and PVA concentration (A), oil to water ratio and GMS quantity (B) and PVA concentration and oil to water ratio (C).

Preparation of tripalmitin based lipospheres: Design of the Experiment (DoE) approach : The DoE approach represents a statistical investigation in which the effects of multiple factors are investigated simultaneously. In a full factorial design the treatments consist of all combinations that can be formed from the different factors, deriving from the number of factors and levels factor tested, while in a fraction factorial design only a fraction of all treatments is included in the experiment. This last design offers a rational approach that enhances the value of the research, reducing the number of experiments and providing much more information about the effects of different variables and their possible interactions.

In particular we performed a randomized central composite face-centered design (CCF) consisting of 17 runs, including three center points in order to have an estimation of the experimental error.
In this way, we obtained both a non linear response and a response surface modelling (RSM).
The MLR equation for the responses of the chosen model is below reported:

$$yi = \text{Constant} + A_1F + A_2P + A_3H + A_4F_2 + A_5P_2 + A_6H_2 + A_7FP + A_8FH + A_9PH$$

where Constant is the mean of runs and Ai the regression coefficients of the factors and their interactions.

The parameters varied were: (a) the percentage of glyceryl monostearate, (b) the stabilizer concentration and (c) the oil to water ratio.

Observing the results reported in Fig. 2 right, it is evident that the parameter having the greatest influence on the lipospheres size and size distribution is the oil to water ratio, while the stabilizer concentration resulted to be the factor with the lowest effect on lipospheres. These results were clearly illustrated in three-dimensional graphs that respectively show the influence of factors on liposphere mean diameter and on the relative standard deviations. The factorial design analysis allowed the selection of the following preparation parameters that resulted in the formation of lipospheres with a spherical shape, narrow size distribution, smooth surface and small amounts of aggregates; glyceryl tripalmitate/glyceryl monostearate 80/20 (%, w/w) as lipid matrix; stirring speed 8000 rpm; PVA concentration 1.0 % (w/v); oil to water ratio 0.04 % (w/w).

Preparation of cationic and drug containing lipospheres
Once the best preparation parameters were selected for neutral lipospheres, we produced cationic (using different cationic surfactants: $DDAC_{18}$, $DDAB_{16}$, $DDAB_{12}$and $C_{18}C_{18}VBr_2$) and drug containing liposferes (Kynurenine, angelicin, dexamethasone, mithramycin).

REFERENCES

- Cortesi R. et al. *Production of lipospheres as carriers for bioactive compounds*, Biomat., 23 283–2294.