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Research paper

Microemulsion and diafiltration approaches: An attempt to maximize the global yield of DNA-loaded nanospheres

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Abstract

The yield of DNA-loaded nanospheres in its widest definition includes encapsulation efficiency and the integrity of the loaded molecules plus the production yield of fabricated nanospheres. The former aspect could be considerably improved by adopting the microemulsion concept to enhance the stability of the primary emulsion during the preparation of nanospheres by the double emulsion solvent-removal method. The droplet size of the mentioned emulsion was monitored by means of photon electron correlation spectroscopy and could serve as an index for emulsion fineness and stability. DNA stability as a function of applied mechanical stress was monitored by horizontal agarose gel electrophoresis. The impact of the primary emulsion on nanosphere porosity was assessed as well.

Regarding the second aspect of the global yield of nanospheres, i.e. production yield, a modified diafiltration technique was adopted for the washing and recovery processes in comparison with the traditional and for the conservation of particle size characteristics of the recovered nanospheres.

Keywords: Primary emulsion; Microemulsion; Encapsulation efficiency; Gel electrophoresis; Diafiltration; Ultracentrifugation; Global yield; Porosity

1. Introduction

Macromolecules, such as proteins, peptides, DNA plasmids and oligonucleotides, originating from the biotechnology procedures; represent a major concern in new therapeutic trend lines. Being unstable, these molecules need to be protected from enzymatic degradation in biological environment. Moreover, they need appropriate carriers for their delivery to be able to cross biological barriers and, if possible, to be targeted to their specific sites of action [1,2].

In this respect, polymeric biodegradable microparticles may represent attractive carriers for many complex molecules and particularly for gene delivery, providing many advantages over viral vectors, such as safety, relative ease of large-scale production, and possible tissue specific targeting [3–5]. Many biologically-safe, biocompatible, biodegradable polymers are now available to prepare such carriers, especially from the polyester series, namely. polylactide (PLA) and polylactide-co-glycolide (PLGA), which have been evaluated extensively [6]. Despite their obvious advantages, particles made from these polymers, once injected, are liable to be rapidly recognized and taken up by the phagocytic system of the body, then rapidly removed from the circulation, as a result of their high negative zeta potential. Several attempts have been made to attribute stealth behaviour to these carrier particles by incorporating hydrophilic groups in the polymeric structure used in their production [7,8]. One of the most successful attempts in this respect is the multiblock copolymer composed of PLA and polyethylene glycol (PEG) (PLA-PEG-PLA)*n*, where the linking of these units via covalent bonding creates stronger attachment of the PEG moiety and is thus not removable by successive washing of microparticles during manufacture [9]. It is thought that such copolymers end in a PEG brush coating on the resultant particle surface and that such dynamic molecular shielding ensures the electrical neutrality required to mislead the MPS prolonging the half life of the injected microparticles in the blood circulation and allowing them to reach the target site [10].

Perhaps the most popular method used of microencapsulating such hydrophilic macromolecules in polymeric carriers is the double emulsion, solvent-evaporation technique because its relative ease, and the possibility of adaptation to obtain particles in the nanometric range, namely, nanospheres [11–14].

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This latest advantage is of special interest whenever gene delivery is required where average carrier particle size below 1 μm seems crucial to ensure successful cellular transfection [15]. Briefly, this procedure consists of two rounds of homogenisation to obtain water in oil in water (w/o/w) double emulsion. After evaporation of the organic solvent and solidification of the microparticles, they are washed with distilled water by successive centrifugation and redispersion cycles. Finally, the redispersed product is usually lyophilised. It is quite evident that DNA molecules are vulnerable to a considerable degree of physical stress due to the mechanical shear applied, especially during the acquisition of the primary emulsion, which could seriously affect their integrity [16]. Besides, nanospheres prepared by the above described technique may generally show other common drawbacks, such as low encapsulation efficiency, aggregation, and low production yield of the collected final product.

Adopting calf thymus DNA as a model macromolecule of biological origin, the goal of the present work is to maximize the global yield of DNA-loaded nanospheres. This means that we have to proceed in two main ways: first, the optimisation of DNA encapsulation efficiency in nanospheres without affecting its stability; second, enhancing the production yield of DNA-loaded nanospheres, rendering the whole procedure worthy for up-scaling and subsequent large-scale production while conserving the particle size characteristics of the prepared nanospheres.

The stability of the primary w/o emulsion seems crucial for encapsulation efficiency, as reported by many workers [17–19]. We have shifted to the microemulsion concept with the aim of monitoring and controlling such emulsions. Indeed, besides the much smaller droplet size in microemulsions compared to regular ones, their relative stability and ease of formation represent strongly-needed features for our purpose [20,21]. Once attributing such features to the primary emulsion, they could significantly minimize migration of the internal aqueous phase containing DNA towards the external aqueous phase during subsequent formation of the multiple emulsion. The loss of DNA molecules in this later phase could, therefore, be reduced. Moreover, such microemulsions could be acquired with a low extent of mechanical shear, which is in itself a major advantage while manipulating fragile macromolecules such as DNA.

For the second aim, the successive centrifugation/redispersion cycles which seem to induce significant aggregation and/or agglomeration of nanospheres, will be substituted by dialysis under reduced pressure, that is, a modified diafiltration technique alternative to cross-flow filtration [22]. This technique will be compared with the traditional washing method in terms of production yield, conservation of nanosphere particle size and washing efficiency for the product.

2. Materials and methods

2.1. Materials

PLA (M_w 50,000) and (PLA-PEG-PLA)*n* multiblock copolymer (M_w 16,000) were synthesized in our laboratory. Calf thymus DNA, as sodium salt, was purchased from Sigma-Aldrich (St Louis, MO, USA) as well as Tris-EDTA buffer. Polyvinyl alcohol (PVA) M_w 9000–10,000, 80% hydrolysed were obtained from Aldrich Chemical Co (Milwaukee, USA), Inc. whereas span 80 was supplied by Fluka Chemika (Buchs, Switzeland). All organic solvents were of analytical grade and supplied by Laboratoire Mat (Beauport, Que., Canada).

2.2. Study of DNA-loaded w/o primary emulsion

As an attempt to monitor the primary w/o emulsion required for the preparation of nanospheres by the double emulsion solvent-evaporation method, a series of DNA-loaded emulsions were prepared by vortexing 500 μl aliquots of aqueous DNA solutions for 60 s in a set of organic phase samples. Each organic phase consisted of 15 ml of dichloromethane (DCM) eventually containing different concentrations of a given surfactant/cosurfactant combination according to a predetermined schedule. Span 80 was chosen as a model lipophilic surfactant for all of the present study, whereas different amphipathic cosolvents were adopted as cosurfactants, namely, the aliphatic medium-chain length alcohols *n*-butanol and *n*-pentanol, as well as ethyl acetate.

The acquired emulsions were kept in a desiccator under reduced pressure (10 psi) for 1 min to remove entrapped air bubbles prior to droplet size determination. The latter was achieved after an equilibration period of 5 min, using photon electron correlation spectroscopy (Nanosizer N4 Plus, Coulter Electronics, Hialeah, FL, USA), where the angle of measurement was 62.5°, run time was 300 s, and temperature was 20 °C The refractive index as well as the viscosity for each organic phase were determined and taken into consideration while running the measurement.

2.3. Effect of mechanical shear on DNA stability

Aliquots of aqueous DNA solutions were preliminarily dispersed by vortexing for 60 s; then each sample was subjected to homogenisation in a high-shear turbine mixer, for a definite period of time ranging from 30 to 180 s. Three different sets of emulsions were tested according to organic phase composition, namely, DCM without additives, DCM containing a span 80/*n*-pentanol combination, and, finally, span 80/*n*-pentanol in DCM associated with 500 mg PLA.

DNA was then recovered by extracting the tested emulsions with 10 ml TE buffer, pH 8, employing end-to-end rotation for 3 h. The aqueous phase was subsequently separated by centrifugation at 14,000 rpm for 10 min. Finally, aliquots of the supernatants containing DNA were pipetted out and subjected to 0.8% agarose horizontal gel electrophoresis for DNA stability assessment.

2.4. Nanospheres preparation

An adjusted double emulsion, solvent-evaporation method was adopted for the preparation of all nanosphere batches tested in our work. Briefly, 500 mg of PLA or a (1:1 w/w) PLA-

multiblock polymer blend was dissolved in 15 ml of DCM, forming the organic phase, into which 0.5 ml of an aqueous solution of DNA in TE buffer, pH 8, was vortexed for 60 s. This dispersion was immediately homogenized for a 30-s period in the turbine mixer to obtain the primary emulsion. The organic phase may incorporate a calculated amount of span 80 either associated or not with a cosurfactant.

The primary w/o emulsion was then gently syringed into 100 ml of 0.5% w/v PVA aqueous solution containing 10% w/v sucrose, while emulsification was being achieved by means of high pressure homogenisation (Emulsiflex C30, Avestin, Ottawa, ON, Canada) at 10,000 psi for 3 min in to obtain multiple w/o/w emulsion. The latter was collected, its volume was adjusted to 250–300 ml with the external aqueous phase, and, finally, it was stirred for 5 h under reduced pressure to allow the extraction and eventually the evaporation of organic solvent(s) and, hence, the subsequent solidification of nanospheres.

2.5. Recovery of nanospheres and the evaluation of PVA clearance

For this study identical batches of DNA-loaded nanospheres were prepared by PLA—(PLA-PEG-PLA)*n* multiblock (1:1) polymer blend as mentioned before.

2.5.1. Ultracentrifugation

Prepared nanospheres were washed and collected by five successive ultracentrifugation/redispersion cycles in deionised water at 41.340 G for 1 h each at 4 °C (Sorvall Evolution, Kendro laboratory Products, Newtown, CT, USA), where PVA concentration was traced in the supernatant after each run by measuring the intensity of the green colour developed after complex formation with iodine and boric acid [23]; then, the values were calculated with reference to a standard calibration curve. Thereafter, the obtained precipitate was directly stored at −20 °C or redispersed in water, quenched in liquid nitrogen and lyophilised prior to storage for further investigation.

2.5.2. Diafiltration

Three identical batches were prepared separately, then subjected to dialysis under reduced pressure in order to get rid of PVA via successive washing/concentration cycles in deionised water. For this purpose a specially-designed quick-fit, all-glass assembly was adopted (Fig. 1). It consists of a glass tank to be filled with the dialyzing solution (0.5% w/v of sorbitol), along with a 3-necked glass cover that could be connected to a vacuum pump. Each Spectra® cellulose ester dialysis tubing (M_w Cut-off 50 kDa) was mounted from one end to the necks of the cover while the other end was closed. The pending tubes were then completely immersed in the dialysis medium in the glass tank, and the cover was firmly fitted on its top. Afterwards, the equipment was totally filled with the solution to expel air from the inside, and a vacuum pump was finally connected via a side arm in the cover. Prepared nanosphere dispersions were subsequently installed in the dialysis bags where they could be dialysed continuously,

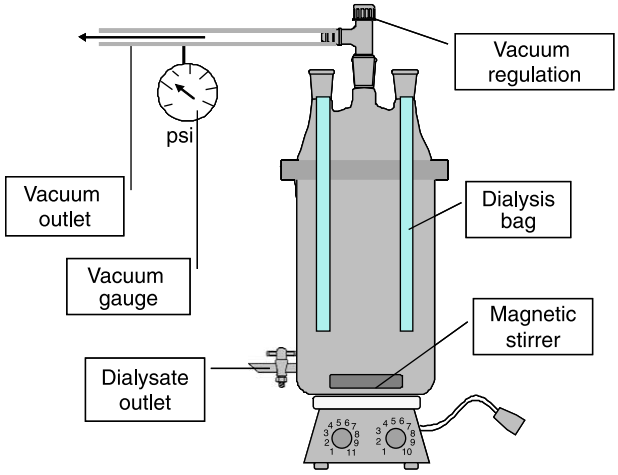

Fig. 1. Schematic representation of the quick-fit diafiltration assembly used for the washing and concentration of nanosphere dispersions.

as mentioned above, taking into consideration the substitution of the dialysis medium with fresh medium at regular time intervals. The PVA concentration was measured regularly in the aqueous phase of the dispersion by the colorimetric method noted previously.

2.6. Determination of DNA encapsulation efficiency and particle size of nanospheres

The extraction procedure was performed according to a published method with little adaptation [24]. A predetermined weight (∼10 mg) of freeze-dried nanospheres was resuspended in 3 ml TE buffer in screw-capped vials; then, 2 ml chloroform was added to solubilize the particles. DNA extraction into the aqueous phase was facilitated by end-over-end rotation of the vials as described above. After centrifugation, 500 μl of the aqueous supernatant was gently aspirated, and DNA was quantified by first derivative UV spectrophotometry at 280 nm, where concentration was calculated with reference to a standard calibration curve. The efficiency of encapsulation was computed as a percentage of encapsulated DNA relative to the initial load after weight correction of the tested amount of nanospheres for that of the starting materials. All experiments were performed at least in duplicate.

Particle size of the fresh nanospheres was determined by photon electron spectroscopy, using the same running parameters mentioned before. The mean of at least five readings was calculated.

2.7. Production yield of nanospheres

This was calculated as a percentage weight of the lyophilised nanospheres with reference to the starting polymeric materials. For the dialyzed batches, the diafiltration procedure was continued until no PVA could be detected in the dispersion; then, the latter was allowed to concentrate to a volume of 25–30 ml. The concentrated batches were circulated in a standard dialysis unit (cross-flow cell), deployed normally

for medical purposes such as haemodialysis, by means of a peristaltic pump, while a counter-current of deionised water was passed inside the cartridge shell under reduced pressure. Such a procedure aimed to get rid from sorbitol that was infiltrated during diafiltration from the dialyzing solution. Complete elimination of the latter was then confirmed by micro-osmometry using μOsmette™ (Precision Systems, Inc., MA, USA) For this purpose, aliquots of the dispersion liquid were obtained by microcentrifugation before and during the washing procedure, and their osmolarity was measured by means of the uOsmette instrument. The osmolarity of the dispersion liquid before the washing procedure was 28 mOsm/l and the procedure was continued until its osmolarity was matched with that of deionised water, meaning zero. Finally, the clean dispersion was freeze-dried as usual.

2.8. Particle size distribution of nanospheres according to different recovery procedures

Particle size distribution of nanospheres was computed by the size distribution processor algorithm provided by the instrument software. The analysis results were plotted as percentage mean values of the selected particle size populations in the form of histogram. Nanospheres coming out of different recovery procedures, namely, ultracentrifugation, diafiltration, as well as freeze-drying, were evaluated for their particle size distribution and compared with freshly prepared nanospheres. Nanospheres aggregated by ultracentrifugation and/or lyophilization were redispersed by vortexing in deionised water for 1 min followed by ultra-sonication for 45 s before measurement.

2.9. Surface area and porosity of the nanospheres

Total surface area and porosity of the freeze-dried nanospheres were determined by the nitrogen adsorption and desorption technique using an automated gas sorption system (Autosorb-1™, Quantachrome Corporation, FL, USA). In short, 100–150 mg of nanospheres were placed in glass sample holders and outgazed at room temperature (20 °C) for 3 h before analysis. Sample and reference tubes were then immersed in liquid nitrogen at −196 °C and sorption isotherms were obtained from the volume of nitrogen (cc/g) adsorbed onto the surface of the nanoparticles as a function of relative pressure. Surface area was calculated by the Brunauer–Emmett–Teller (BET) method [25] using five adsorption points in the P/P_0 range of 0.05–0.3. Pore size distribution was calculated according to the Barret–Joyner–Halenda method [26].

3. Results and discussion

Several authors have reported the utility of surfactants for enhancing the stability of the primary emulsion as a first criterion in the microencapsulation process, when the double emulsion, solvent-evaporation technique is adopted [17–19]. In this respect, microemulsion concept seems attractive regarding the much finer dispersion that could be obtained, its higher stability, and the lower energy input required for emulsification, compared to regular emulsion [20]. Besides, some particular advantages of our application is that w/o microemulsions need little cosurfactant to be prepared as well as they show great stability relative to o/w emulsions [27,28].

3.1. Microemulsification studies for the primary w/o emulsion

Micellar dispersion of aqueous solution in organic phase could be brought to microemulsion through the addition of cosurfactants; generally, medium-chain alcohols are good candidates for this purpose. Hence, we have investigated the impact of span 80 in association with selected cosurfactants, on droplet size in a series of DNA-loaded w/o emulsions where DCM serves as the organic dispersing phase. By such an attempt, we try to gain insight into the primary emulsion to monitor and optimise the conditions of its formation.

The 3D response curves (Figs. 2–4) reveal the dependency of droplet size on both surfactant and cosurfactant proportions. Span 80 alone induces a sharp reduction in droplet size as a function of its concentration until a certain value is attained. However, more surfactant does not induce further decreases in droplet size. This result is in good accordance with the work of El-Mahdy et al. [29] who explained that the mean droplet size of the dispersed phase in w/o emulsion has already been stabilized by a sufficient amount of span 80 that forms a thin film at the interface between the droplets and the oily phase, enough to prevent their coalescence and subsequent droplet size enlargement. Alternatively, excess surfactant has been shown to elicit an apparent increase in droplet size which may be attributed to the elongation of formed micelles with the possibility of their subsequent orientation to bring closely packed arrays.

On the other hand, the coupling of span 80 with either *n*-butanol or *n*-pentanol leads to more pronounced droplet size reduction in the investigated emulsion. From Fig. 2, the minimal droplet size reached was 101.3 nm when 0.4% w/v of span 80 was associated with 13.3% v/v *n*-butanol, whereas 0.6% w/v of the surfactant along with 20% v/v of *n*-pentanol diminished the droplet size to 93.6 nm (Fig. 3). Nevertheless, further increase of the cosurfactant proportion induced visible precipitation of DNA from the aqueous phase, with an apparent increase of droplet size. This event could be attributed to the salting out effect evoked by the aliphatic alcohol once its concentration is raised in the internal aqueous phase via partitioning from the interface after the saturation of the latter by cosurfactant molecules.

As for ethyl acetate, Fig. 4 shows only a limited reduction of droplet size when the latter was associated with span 80. The least average droplet size attained was 285.9 nm at 0.3% w/v of span 80 and 20% v/v of ethyl acetate.

The advantage of surfactant/cosurfactant blending is quite evident. The non-ionic surfactant is the main solubilizer, and the cosurfactant, when added gradually can adjust the HLB of the mixture to an optimum value, i.e. this will result in the reduction of the interfacial tension to the minimum [27,28], in

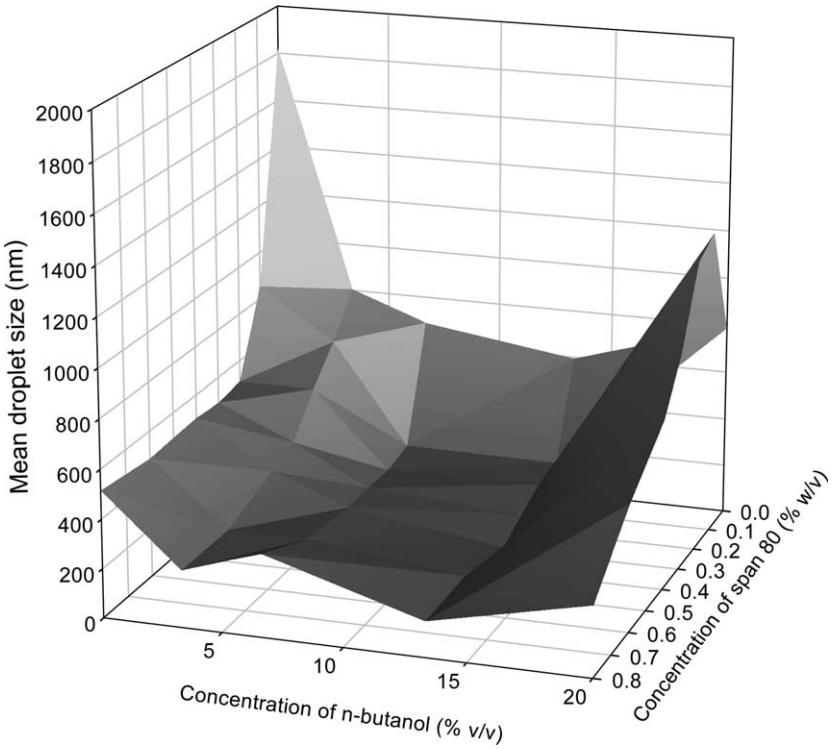

Fig. 2. Surfactant/cosurfactant (span 80/*n*-butanol) response curve for droplet size in w/o emulsion system.

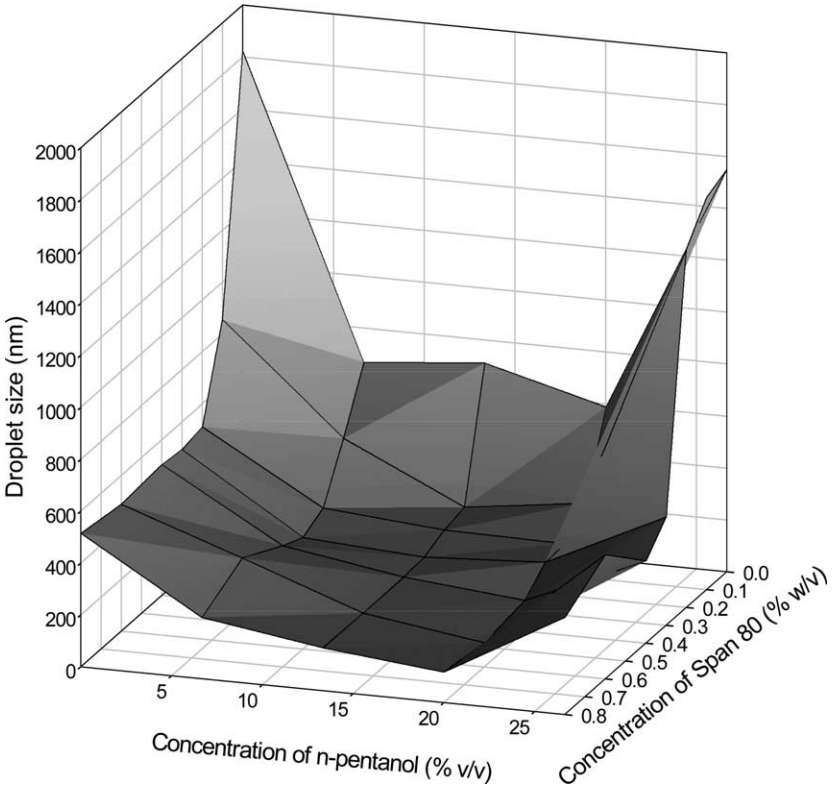

Fig. 3. Surfactant/cosurfactant (span 80/*n*-pentanol) response curve for droplet size in w/o emulsion system.

other words, attaining solubilization or microemulsification. The cosurfactant acts through its padding effect [30], which means packing its molecules at the interface between the surfactant molecules, separating the polar heads of the latter, and thus preventing their mutual repulsion. Such effect ensures a low free energy of the system and, consequently, can greatly contribute to its stability. Moreover, the cosurfactant migrates to regions of strong curvature, enhancing the flexibility of the

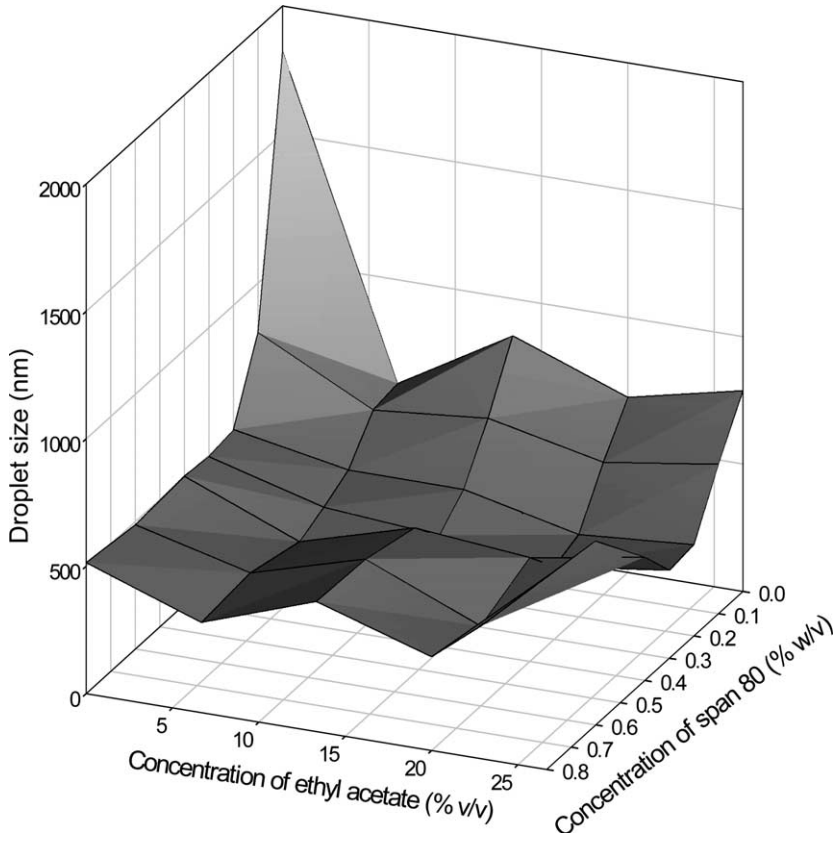

Fig. 4. Surfactant–cosurfactant (span 80—ethyl acetate) response curve for droplet size in O/W emulsion system.

interface between the phases and the formation of bicontinuous regions [31]. This feature may explain the observed suppression of droplet size growth that usually accompanies the high concentrations of the surfactant, once this latter is associated with a cosurfactant. Indeed, combining *n*-butanol or *n*-pentanol with span 80 seems able to delay the sequence of micellar elongation and packing that may ultimately result in the rigid lamellar phase.

The limited ability of ethyl acetate to reduce the droplet size of the dispersed phase compared to medium-chain aliphatic alcohols could be understood in view of the padding concept and the molecular configuration of these cosurfactants. As shown in the schematic representation of the droplet surface in Fig. 5, the polar head in aliphatic alcohols is aligned nearly straightforward with the main chain of the molecule; this will permit the molecule to be easily packed on the interface along with the surfactant molecules with a minimum need of free surface area, i.e. without the necessity of bending the curved interface. Once embedded in the internal aqueous phase, the lone pair of electrons on the oxygen atom can easily form hydrogen bonding with water molecules. Oppositely, for ethyl acetate, the dipolar momentum of the molecule is not aligned with its geometric axis as the polar carbonyl group is located between two carbon chains. Such a configuration requires a larger free surface area on the interface so as to enable the polar group to get access to the internal aqueous phase. In other words, the padding process will be quite difficult unless the radius of the curvature at the interface increases, which means droplet size enlargement.

3.2. DNA stability in response to mechanical shear

As seen in Fig. 6(A), non-treated DNA is intact as is DNA subjected to 60-s vortexing with the aim of dispersing it in the organic phase. On the other hand, all DNA samples treated by high-speed stirring for homogenisation in DCM are undergoing

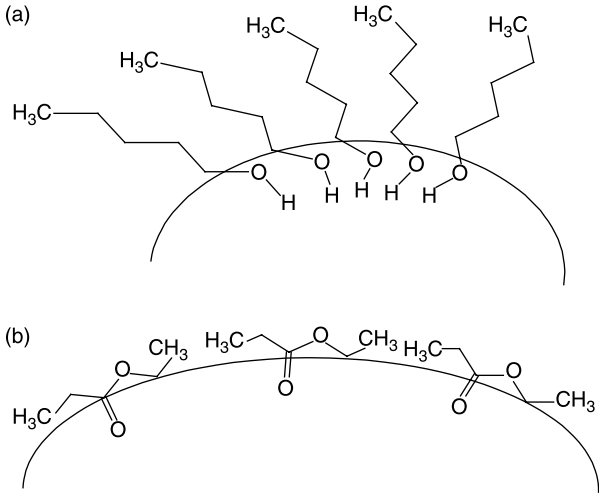

Fig. 5. Schematic representation of different cosurfactant molecule organizations at the w/o interface in the primary emulsion. (A) aliphatic alcohol, (B) ethyl acetate.

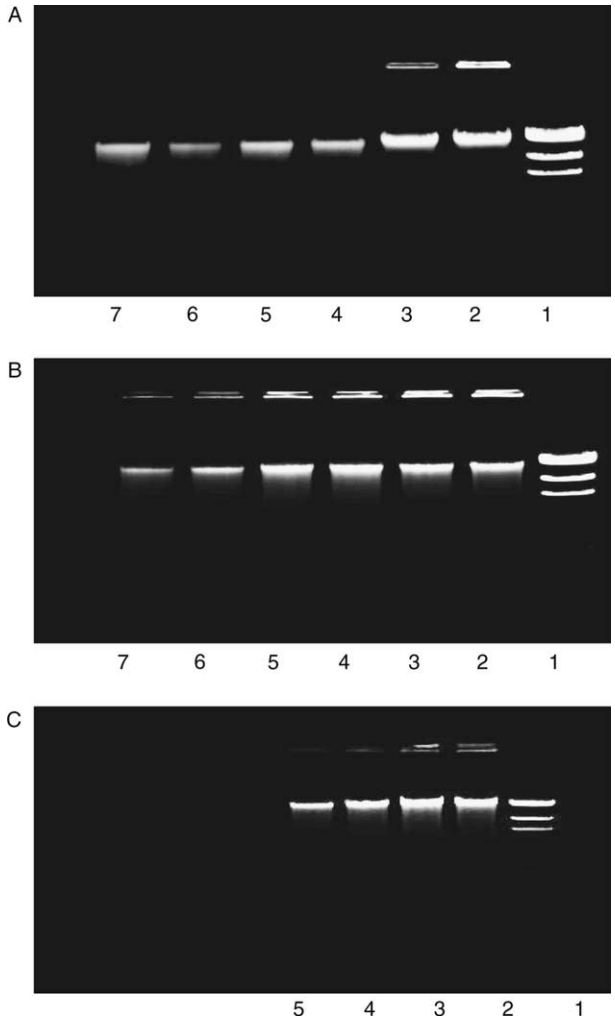

Fig. 6. (A) Stability of DNA mechanically dispersed as an aqueous solution in DCM: 1, Ladder; 2, Standard untreated DNA, Samples tested according to the homogenisation period; 3, 0 s; 4, 30 s; 5, 60 s; 6, 90 s; 7, 120 s. (B) Stability of DNA mechanically emulsified as an aqueous solution in DCM without polymer with the aid of a surfactant/cosurfactant mixture (span 80/*n*-pentanol): 1, Ladder; 2, Standard untreated DNA, Samples tested according to the homogenisation period; 3, 0 s; 4, 30 s; 5, 60 s; 6, 90 s; 7, 120 s. (C) Stability of DNA mechanically emulsified as an aqueous solution in DCM containing polymer with the aid of a surfactant/cosurfactant mixture (span 80/*n*-pentanol): 1, Ladder; 2, standard untreated DNA; tested samples according to the homogenisation period; 3, 60 s; 4, 120 s; 5, 180 s. (A–C). Agarose horizontal gel electrophoresis of DNA samples subjected to mechanical shear under different conditions for stability assessment. All test samples were preliminarily dispersed by vortexing for 1 min prior to homogenisation for different periods of time.

progressive degradation, as reflected by the gradual fading of their respective fluorescent bands as a function of mixing time.

Alternatively, it could be depicted that DNA emulsified in the organic phase with the aid of surfactant/cosurfactant combination exhibits much better tolerance to the applied mechanical shear. As shown in Fig. 6(B), the intensity of lane 3 is comparable to the standard which signifies that up to 1 min of homogenisation is not liable to affect DNA integrity in such a system.

It is known that hydrophilic macromolecules, such as proteins, enzymes, and DNA, possess some interfacial characteristic [32,33]; therefore in the absence of surfactant, DNA molecules tend to be more or less adsorbed at the interface between the aqueous and the organic phases and become therefore vulnerable to physical stress induced by severe mixing once applied with the aim of emulsification. The enhanced protection seen in the presence of surfactant/cosurfactant combination could be attributed to the arrangement of their molecules at the w/o interface while displacing DNA molecules towards the bulk of the dispersed aqueous phase which, in turn, renders them less subject to the mechanical shear acting at the interface. Moreover, the extremely reduced interfacial tension obtained in the adopted microemulsion system could considerably buffer the shear effect.

Fig. 6(C) shows that the addition of the polymer to the precedent system can provide additional protection to DNA. Lane 4 corresponding to 2-min homogenisation exhibits only a little faintness in its luminescence relative to the preceding lane equivalent to 1-min mixing. Certainly, the incorporation of the polymer in the primary emulsion can raise the viscosity of the organic phase, and this effect seems to mitigate the mechanical shocks transmitted to DNA molecules.

Compared to other methods used to protect DNA from physical stress during microencapsulation [16,34,35], the present technique appears to be quite useful, taking into consideration that the required energy input to form the primary emulsion could be significantly reduced due to the spontaneous nature of microemulsion formation.

3.3. DNA encapsulation efficiency and the particle size of nanospheres

The encapsulation efficiency of DNA incorporated in nanospheres depends strongly on the polymer. By comparing Tables 1 and 2, it could be easily deduced that all nanosphere batches prepared from the PLA-multiblock blend give higher encapsulation values than those made of PLA alone. This latter yielded as much as 66.36% of DNA encapsulated relative to the initially loaded amount, whereas a blend of PLA and multiblock could raise this value up to 79.71%. Such a fact could be interpreted in light of the presence of hydrophilic domains attributed to the PEG moieties in the multiblock polymer. During preparation of the primary w/o emulsion, these domains could contribute significantly to the stabilization of the latter which will be positively reflected in the DNA amount encapsulated in the end product [36]. Moreover, it is obvious that nanospheres prepared with polymer blend have larger particle size compared to those made from PLA alone. This could be due to the higher amount of DNA-loaded in the former preparations as more volume of the aqueous buffer containing DNA should be incorporated in the primary w/o emulsion which, in turn, has to be emulsified in the external aqueous phase to finally give solidified nanospheres.

On the other hand, the microemulsion technique can greatly improve DNA encapsulation efficiency in prepared

Table 1
Primary emulsion composition and characteristics of DNA-loaded nanospheres prepared from PLA by the double emulsion solvent-evaporation method

Cosurfactant	Proportion (%v/v)	Span 80 (%w/v)	Mean diam. (nm)	Loading efficiency (%)	Production yield (%)
–	–	–	170	17.58	29.6
–	–	0.4	193.6	34.06	40.3
Acetone	33.3	0.4	168.0	12.95	36.9
n-Butanol	16.6	0.4	170.5	66.36	52.5
n-Pentanol	20	0.6	196.1	53.81	58.0
Ethyl acetate	20	0.3	163.4	37.64	49.2

Table 2
Primary emulsion composition and characteristics of DNA-loaded nanospheres prepared from 1:1 blend of PLA and (PLA-PEG-PLA)*n* multiblock copolymer by the double emulsion solvent-evaporation method

Cosurfactant	Proportion (%v/v)	Span 80 (%w/v)	Mean diam. (nm)	Loading efficiency (%)	Production yield (%)
			240.9		55.1
–	–	0.4	350.6	53.19	65.8
n-Butanol	16.6	0.4	266.9	75.53	60.5
n-Pentanol	20	0.6	290.7	79.71	51.3
Ethyl acetate	20	0.3	203.1	58.91	45.5

nanospheres regardless of the polymer used. In this respect, the aliphatic alcohols *n*-butanol and *n*-pentanol as cosurfactants have a considerable impact on such a critical aspect. Compared to the encapsulation values mentioned above that have been obtained with the aid of either of the tested aliphatic alcohols, only 17.58% and percent could be attained when PLA or its blend with the multiblock were employed, respectively.

As expected, improvement was limited when ethyl acetate was used as cosurfactant with span 80. This fact may be easily attributed to the minor effect of this cosurfactant on droplet size of the w/o emulsion, as seen above. In general, the obtained DNA encapsulation results correlate well with those obtained from microemulsion studies. Indeed, average droplet size of the primary emulsion could be considered a valuable index for the fineness and stability of the latter. These two aspects seem crucial for the retention of any loaded hydrophilic ingredient, such as DNA, in nanospheres acquired by the double emulsion technique.

3.4. Production yield of recovered nanospheres

The average yield of the dialyzed batches of nanospheres was 83.9 ± 6.1 (mean $\pm$ SD for $n = 3$), whereas the yield values for nanosphere batches recovered by ultracentrifugation ranged from 29.6 to 65.8% (mean $\pm$ SD 49.4 ± 10.8) as shown in Tables 1 and 2. It should be noted that for each lyophilised batch of nanospheres, unwashed PVA was quantified according to a published method [37], and its residual amount in the final product was taken into account while calculating yield.

We thought that yield values obtained by ultracentrifugation are not surprising, since much of the smaller nanospheres are not liable to precipitate despite the high centrifugal force adopted during successive washing runs of the product. Besides, a part of the precipitated nanospheres is highly subjected to loss when the supernatant is discarded even with high-care handling which is, finally, a human factor. It should be noted that increasing the centrifugal force beyond this value would encounter several limitations and may not be the ideal solution. Alternatively, the production yield gained by the diafiltration technique is considerably high relative to the former values, and may be acceptable for upgrading the production scale. Here, the observed loss of nanospheres may be attributed to the production procedure itself rather than the recovery step.

3.5. PVA clearance according to the recovery method

PVA clearance during the course of washing the nanospheres is shown in Fig. 7. It is clear that PVA elimination by the ultracentrifugation procedure is much faster than that achieved with diafiltration. However, it could be noted that by increasing the surface area of the dialyzing membrane, the process can be significantly enhanced. Unfortunately, for our specially designed instrument, the possibility of enhancing this parameter was limited; nevertheless, it could easily be improved for scaling up purposes.

It is worthy mentioning that we have tried the capillary polysulfone fibber ultrafiltration unit (miniKross module, Spectrum Laboratories, Inc., CA, USA) providing a 1050-cm^2 membrane surface area, (molecular cut-off 400 kDa) for nanospheres washing. Freshly prepared nanosphere dispersion were allowed to circulate through the capillary fibbers by means of peristaltic pump while a counter current of deionised water was running tangentially to the external surface of the capillaries at a high flow rate. Amazingly, the PVA suffered very limited permeation via the membrane fibber wall, and its elimination from the batch was practically impossible even though that we strictly followed the manufacturer's instructions concerning the membrane promotion prior its use.

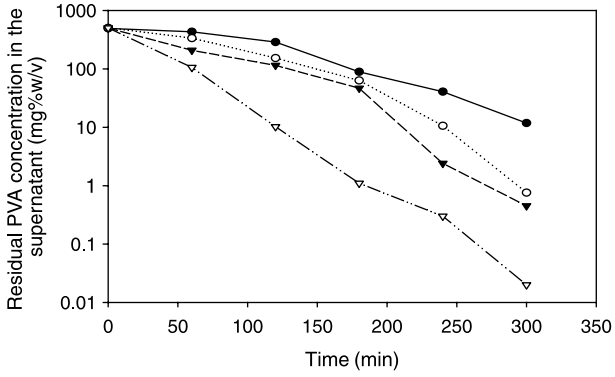

Fig. 7. Clearance of PVA from aqueous dispersions of nanospheres during the washing procedure by diafiltration and ultracentrifugation. Closed circles: 157.14 cm^2, Open circles: 314.3 cm^2, Closed triangles: 471.4 cm^2, Open triangles: ultracentrifugation at 41.314 G.

Finally, we thought that the permeability of the cellulose ester membrane to PVA is quite reasonable and that washing of the stabilizer could be practically achieved at an acceptable rate once certain factors are taken into account, such as adequate membrane surface area (surface of exchange), rate of renewal of the dialyzing medium, and appropriate molecular cut-off of the membrane.

3.6. Particle size distribution of nanospheres as a function of the recovery procedure

As a result of ultracentrifugation, the nanospheres get together under the huge centrifugal force applied forming, tightly bound aggregates and/or agglomerates. To redisperse the particles, many workers may use surfactants frequently associated with ultrasonication to recover the particle size of the freshly prepared nanospheres. From a practical point of view, this may not be the ideal method of recovering the most distinguishing feature of such carriers, their particle size. It would be better to attempt to conserve the original particle size of the nanospheres by adopting a tender washing/recovery technique rather than using such a harsh procedure for their recovery trying, thereafter, to revert the undesired resulting changes by seeking another drastic tool, such as ultrasonication, that may harm fragile encapsulated biomolecules, and most often, with the use of some additive rendering the washing procedure meaningless [38].

In the present work, it should be noted that the dialyzed nanospheres were not subjected to any treatment after the decongealing of their concentrate except for vortexing for a few seconds prior to measurement, whereas ultracentrifuged and lyophilised nanospheres were subjected to redispersion, as mentioned earlier to break down their aggregates.

In Fig. 8 (A–D), particle size distribution patterns are given as a function of different recovery procedures. After PVA removal, the concentrated dispersions of nanospheres, nanospheres precipitated by ultracentrifugation and lyophilised nanospheres were stored at −20 °C for 2 weeks before particle size analysis. It can be deduced from Fig. 8(A) and (B) that the nanospheres treated by diafiltration did not suffer much alteration in their particle size (313.88 ± 15.6) compared to freshly prepared ones (240.93 ± 15.7). The nanoparticle population at 1 μm seems not to be positively influenced by the recovery procedure; it is rather the population located at

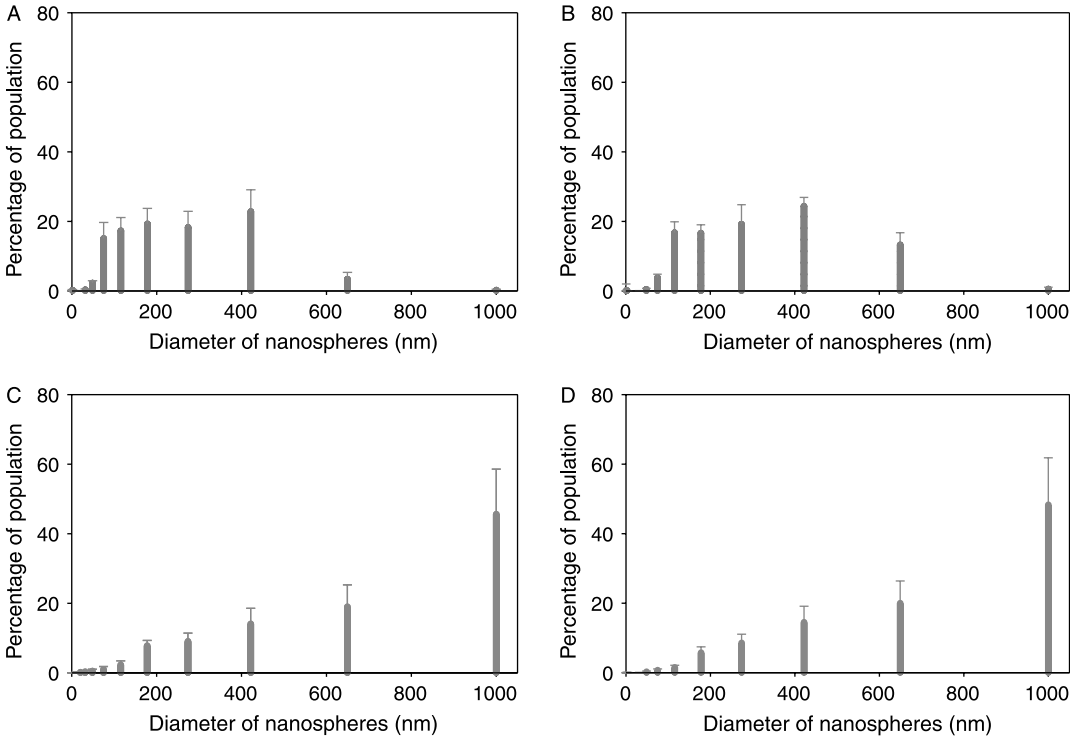

Fig. 8. (A–D). Particle size distribution of DNA-loaded nanospheres before and after different washing and recovery procedures.

645 nm that exhibits an obvious growth on the expense of those having a mean diameter less than 150 nm.

On the other hand, nanospheres, either precipitated by ultracentrifugation or subsequently lyophilised, manifest significant changes in their particle size distribution, most probably because of agglomeration that seems irreversible even after the redispersion procedure (Fig. 8(C) and (D)). In this respect, it is clear that the 1 μm population manifests pronounced inflation whereas all the other groups suffer an obvious decrease in amplitude.

It is worthy to mention also that the stability of DNA encapsulated in the nanospheres under investigation in this section was confirmed through horizontal gel electrophoresis, after extraction of the hydrophilic molecule in aqueous buffer from the DCM-degraded nanospheres, as explained earlier (data not shown). This means that the recovery procedures of the end product used herein have no notorious effect on the DNA integrity.

3.7. Surface area and porosity of the nanospheres

The BET method is based on the adsorption and desorption of nitrogen or krypton gas at the surface and within the pores of nanospheres. Porosity and the surface area parameters of four batches of nanospheres prepared from PLA-multiblock polymer blend (1:1 w/w) are depicted in Table 3. Micropore analysis was performed according to the Dubinin–Radushevich method [39].

It is evident that the average values attributed to pore width mainly reflect mesopores rather than the micropores. Mesopores lying within such a pore width range (3–5 nm) are not likely to reflect the porosity of nanospheres treated in our work, and the contribution of the nanosphere pores appears to be negligible in such a parameter. So, to get a better idea of the porosity of our nanospheres, we can rely on other tabulated parameters (Table 3), namely, average and total micropore volumes, micropore surface area and BET surface area.

The wide variations of these values among the prepared batches of nanospheres well reflect the differences of porosity between them and, hence, could be easily correlated with primary emulsion stability as it was reported by Schugens et al. [40]. For instance, the use of span 80/*n*-pentanol combination which induces the most stable primary emulsion as seen in Section 3.1, achieves the lowest values of the porosity parameters in the obtained nanospheres (batch No. 4). On the other hand, the highest average porosity could be easily attributed to nanosphere batch No. 1, where no surfactant had been used to stabilize the primary emulsion. This seems in good agreement with the relatively coarse primary emulsion produced during the preparation of these nanospheres. Hence, we could safely deduce that the average porosity is in a straightforward relationship with the fineness of the primary emulsion which, in turn, reflects the stability of the latter as mentioned above.

We think that reduced porosity of the nanosphere matrix may more or less contribute to the enhancement of the encapsulation efficiency of hydrophilic molecules by hindering their undesirable release through pores during the solvent removal and washing procedures of the nanospheres.

4. Conclusion

This work first demonstrates that the microencapsulation efficiency of a hydrophilic macromolecule such as DNA in nanospherical carriers could be significantly improved by adopting the microemulsion concept while keeping the average size of the carriers within the acceptable range for cellular uptake and transfection purposes. Certainly, many surfactant/-cosurfactant pairs may be tried; however, we claim that the technique presented for monitoring the primary emulsion has proven quite useful to predict as well as to optimise the conditions required for incorporating biomolecules into nanospheres acquired by the double emulsion technique. Moreover, another crucial requirement that may be fulfilled while adopting this approach is conservation of the integrity of those fragile molecules, since only limited mechanical work should be applied. One important reason that makes calf thymus DNA, a genomic DNA, useful as a model macromolecule in this work is its extreme sensitivity to mechanical shear [41]. If it is possible to conserve this DNA integrity during the microencapsulation procedure by the microemulsion approach, then this latter will more likely succeed in preserving the integrity of many other less fragile macromolecules such as plasmid DNA whenever their microencapsulation is required. Although gel electrophoresis cannot serve as a quantitative method for DNA analysis, it is widely used as practical tool to assess its stability by comparing the intensity of its corresponding lanes. Hence, these two topics, namely, the

Table 3
Porosity and surface area characteristics for PLA-Multiblock nanospheres prepared by the double emulsion technique and having different primary emulsion compositions

Parameters (mean ± SD for $n=3$)	Batch No. 1[a]	Batch No. 2	Batch No. 3	Batch No. 4
Average pore width (nm)	4.549 ± 0.043	4.664 ± 0.295	4.533 ± 0.165	3.684 ± 0.420
Average micropore volume (cc/g)	$5.50\times10^{-3}\pm5.6\times10^{-4}$	$2.04\times10^{-3}\pm6.5\times10^{-4}$	$7.44\times10^{-4}\pm1.1\times10^{-4}$	$1.15\times10^{-4}\pm5.0\times10^{-5}$
Total micropore volume (cc/g)	0.335 ± 0.05	0.255 ± 0.01	0.168 ± 0.02	0.031 ± 0.01
Micropore surface area (m^2/g)	15.49 ± 1.57	5.743 ± 1.82	2.095 ± 0.30	0.324 ± 0.14
BET surface area (m^2/g)	21.767 ± 6.05	18.807 ± 5.59	9.675 ± 0.27	3.423 ± 1.26

[a] According to the composition of the primary emulsion, the code attributed to the prepared batches of nanospheres is: Batch 1, ± No surfactant, no co-surfactant; Batch 2, Span 80, no co-surfactant; Batch 3, Span 80, *n*-butanol; Batch 4, Span 80, *n*-pentanol.

encapsulation efficiency and the stability of the precious therapeutic agent could be just considered as one aspect of the global yield of carriers, the loading yield.

The second aspect is the production yield of the final product, in other words the nanospheres themselves. As it has been shown, diafiltration technique can considerably raise the percentage of the recovered product relative to the starting materials when used for the washing and concentration of the freshly prepared dispersion. Moreover, the particle size characteristics of the carriers have been greatly conserved when compared to ultracentrifugation where these two advantages are missing since the production yield will never be satisfactory for scaling up purposes, besides, the aggregation of nanospheres will discount one important feature of those carriers as it will limit their application [38]. Hence, as it has been suggested before [13], we are in favour of congealing the concentrated nanospheres dispersion at −20 °C after the recovery procedure. The concentration of the medicinal agent could be determined per unit volume of the liquid dispersion and adjusted if needed. Such liquid preparations could be easily adapted to be an injectable dosage form after adjusting the tonicity and sterilization.

References

[1] K. Kawabata, Y. Takatura, M. Hashida, The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake, Pharm. Res. 12 (1995) 825–830.

[2] D. Ferber, Gene therapy, safer and virus free?, Science 294 (2001) 1638–1642.

[3] E. Walter, K. Moelling, J. Pavlovic, H. Merkle, Microencapsulation of DNA using poly (DL-lactide-co-glycolide) stability issues and release characteristics, J. Control. Release. 3 (1999) 361–374.

[4] D. Wang, D.R. Robinson, G.S. Kwan, J. Samuel, Encapsulation of plasmid DNA in biodegradable poly (DL-lactide-co-glycolide) microspheres as a novel approach for immuno gene delivery, J. Control. Release. 57 (1999) 9–18.

[5] J. Eldridge, J. Stoas, J. Meulbrock, J. McGhee, T. Tice, R. Gilley, Biodegradable microspheres as a vaccine delivery system, Mol. Immunol. 26 (1991) 287–294.

[6] D.H. Lewis, Controlled release of bioactive agents from lactide/glycolide polymers in: M. Chasin, R. Langer (Eds.), Biodegradable Polymers as Drug Delivery Systems, Marcel Dekker, New York, 1990, pp. 1–41.

[7] D. Bazile, C. Prudhomme, M.T. Bassoullet, M. Marlard, G. Spenlehauer, M. Veillard, Stealth Me.PEG–PLA nanoparticles avoid uptake by the mononuclear phagocytes system, J. Pharm. Sci. 84 (4) (1995) 493–498.

[8] Y. Nakada, K. Sakurai, M. Horiushi, R. Tudmi, T. Nakamura, Y. Takahashi, Long-circulating nanoparticles using biodegradable ABA triblock copolymers containing poly(lactic acid) A-blocks attached to central poly(oxy ethylene)B-blocks, J. Pharm. Sci. 3 (1997) 1–4.

[9] R. Quesnel, P. Hildgen, Synthesis and characterization of alternate (PLA–PEG–PLA)m multiblock copolymers Proceedings of the 4th World Meeting ADRITELF/APGI/APV, Florence April 8–11, 2002.

[10] Y. Yamamoto, Y. Nagasaki, Y. Kato, Y. Sugiyama, K. Kataoka, Long-circulating poly(ethylene glycol)-poly(D,L-lactide) block copolymer micelles with modulated surface charge, J. Control. Release. 77 (2001) 27–38.

[11] M.M. Iwata, J.W. McGinity, Preparation of multiphase microspheres of poly (lactic acid) and poly(DL-lactide-co-glycolide) containing a w/o emulsion by a multiple solvent evaporation technique, J. Microencapsul. 9 (1992) 201–214.

[12] M.D. Blanco, M.J. Alonso, Development and characterization of protein-loaded poly(DL-lactide-co-glycolide) nanospheres, Eur. J. Pharm. Biopharm. 43 (1997) 287–294.

[13] T. Hao, U. Mckeever, M.L. Hedley, Biological potency of microsphere encapsulated plasmid DNA, J. Control. Release. 69 (2000) 249–259.

[14] A. Lamprecht, N. Ubrich, M. Hombreiro-Perez, C.M. Lehr, M. Hoffman, P. Maincent, Biodegradable monodispersed nanoparticles prepared by pressure homogenization-emulsification, Int. J. Pharm. 184 (1999) 97–105.

[15] Y. Tabata, Y. Ikada, Phagocytosis of polymer microspheres by macrophages, Adv. Polym. Sci 94 (1990) 107–141.

[16] M. Dunne, D.C. Bibby, J.C. Jones, S. Cudmore, Encapsulation of protamine sulphate compacted DNA in polylactide and polylactide-co-glycolide microparticles, J. Control. Release. 92 (2003) 209–219.

[17] Y.F. Maa, C.C. Hsu, Effect of primary emulsions on microsphere size and protein-loading in the double emulsion process, J. Microencapsul. 14 (1997) 225–241.

[18] N. Nihant, C. Schugens, C. Grandfils, R. Jerome, P. Teyssié, Polylactide microparticles prepared by double emulsion/evaporation technique. I. Effect of primary emulsion stability, Pharm. Res. 11 (1994) 1479–1484.

[19] K.J. Zhu, H.L. Jiang, X.Y. Du, J. Wang, W.X. Xu, S.F. Liu, Preparation and characterization of hCG-loaded polylactide or poly (lactide-co-glycolide) microspheres using a modified water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique, J. Microencapsul. 18 (2001) 147–260.

[20] L.H. Block, Pharmaceutical emulsion and microemulsions in: H.A. Lieberman, M.M. Rieger, G.S. Banker (Eds.), Pharmaceutical Dosage Forms: Disperse Systems 2, Marcel Dekker, Inc., New York, 1996, pp. 49–50.

[21] K. Shinoda, H. Kunieda, T. Arai, H. Saijo, Principles of attaining very large solubilization (microemulsion): inclusive understanding of the solubilization of oil and water in aqueous and hydrocarbon media, J. Phys. Chem. 88 (1984) 5126–5129.

[22] E. Allémann, E. Doelker, R. Gurny, Drug loaded poly(lactic) nanoparticles produced by a reversible salting out process: purification of an injectable dosage form, Eur. J. Pharm. Biopharm. 39 (1993) 13–18.

[23] J. Eliassaf, Detection of small quantities of poly(vinyl alcohol) in poly(vinyl alcohol) resins, J. Polym. Sci. B Polym. Lett. Ed. 10 (1972) 697–698.

[24] S.P. Barman, L. Lunsford, P. Chambers, M.L. Hedley, Two methods for quantifying DNA extracted from poly(lactide-co-glycolide) microspheres, J. Control. Release. 69 (3) (2000) 337–344.

[25] S. Brunauer, P.H. Emmett, E. Teller, Adsorption of gases in multimolecular layers, J. Am. Chem. Soc. 60 (1938) 309–319.

[26] E.P. Barrett, L.G. Joyner, P.P. Halenda, The determination of pore volume and area distributions in porous substances. I- Computations from nitrogen isotherms, J. Am. Chem. Soc. 73 (1951) 373–380.

[27] M. Rosoff, Specialized pharmaceutical emulsions in: H.A. Lieberman, M.M. Rieger, G.S. Banker (Eds.),second ed. Pharmaceutical Dosage Forms: Disperse Systems 3, Marcel Dekker, Inc., New York, 1996, pp. 20–24.

[28] L.M. Prince, Microemulsions Theory and Practice, Academic Press, Inc., New York, 1977. pp. 33–49, pp. 79–80.

[29] M. El-Mahdy, E.S. Ibrahim, S. Safwat, A. El-Sayed, H. Ohshima, K. Makino, N. Muramatsu, T. Kondo, Effects of preparation conditions on the monodispersity of albumin microspheres, J. Microencapsul. 15 (5) (1998) 661–673.

[30] D. Oakenfull, Constraints of molecular packing on the size and the stability of microemulsion droplets, J. Chem. Soc., Faraday Trans. 1 76 (1980) 1875–1886.

[31] J. Jouffroy, P. Levinson, P.G. De Gennes, Phase equilibria involving microemulsions, J. Phys. 43 (1982) 1241–1248.

[32] G. De Rosa, R. Iommelli, M.I. La Rotonda, A. Miro, F. Quaglia, Influence of the co-encapsulation of different non-ionic surfactants on the properties of PLGA insulin-loaded microspheres, J. Control. Release. 69 (2000) 283–295.

[33] M. Van de Weert, W.E. Hennink, W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles, Pharm. Res. 17 (2000) 1159–1167.
[34] Y. Capan, B.H. Woo, S. Gebrekidan, S. Ahmed, P.P. DeLuca, Stability of poly (L-lysine)-complexed plasmid DNA during mechanical stress and DNase I treatment, Pharm. Dev. Technol. 4 (1999) 491–498.
[35] S. Ando, D. Putnam, D.W. Pack, R. Langer, PLGA microspheres containing plasmid DNA: Preservation of supercoiles DNA via cryoprotection and carbohydrate stabilization, J. Pharm. Sci. 88 (1999) 126–130.
[36] Y.Y. Yang, J.P. Wan, T.S. Chung, P.K. Pallathadka, S. Ng, J. Heller, POE-PEG-POE triblock copolymer microspheres containing protein. I. Preparation and characterization, J. Control. Release. 75 (2001) 115–128.
[37] S.K. Sahoo, J. Panyam, S. Prabha, V. Labhasetwar, Residual polyvinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, J. Control. Release. 82 (2002) 105–114.
[38] Y.N. Konan, R. Gurny, E. Alléman, Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles, Int. J. Pharm. 233 (2002) 239–252.
[39] M.M. Dubinin, Fundamentals of the theory of adsorption in micropores of carbon adsorbents: characteristics of their adsorption properties and microporous structures, Carbon 27 (1989) 457–467.
[40] C. Schugens, N. Laruelle, N. Nihant, Ch. Gradfils, R. Jérome, P. Teyssé, Effect of the emulsion stability on the morphology and porosity of semicrystalline poly1-lactide microparticles prepared by w/o/w double emulsion-evaporation, J. Control. Release. 32 (1994) 161–176.
[41] M. Schleef, T. Schmidt, Animal-free production of ccc-supercoiled plasmids for research and clinical applications, J. Gene Med. 6 (2004) S45–S53.