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Nanomedicine & Nanotechnology

Swed et al., J Nanomed Nanotechnol 2014, 5:6
http://dx.doi.org/10.4172/2157-7439.1000241

Research Article Open Access

Protein Encapsulation into PLGA Nanoparticles by a Novel Phase Separation Method Using Non-Toxic Solvents

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Abstract

Nanoparticles of biocompatible and biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) are widely used as drug delivery systems for the administration of biomolecules like proteins. The purpose of this work is to validate a novel formulation method by a phase separation phenomenon using the non-toxic solvent glycofurol (GF) in order to encapsulate proteins into PLGA nanoparticles. Nanoprecipitates of a model protein (lysozyme) and a therapeutic protein (TGF-β1) were formed to ensure their stability upon subsequent encapsulation into PLGA nanoparticles. Good encapsulation efficiency was obtained with preservation of the structure integrity and protein bioactivity after encapsulation. PLGA nanoparticles were then characterized in terms of size, zeta potential and morphology. Moreover, residual solvent was quantified and *in vitro* release study of the encapsulated proteins was performed to demonstrate the efficacy of our encapsulation method in drug sustained release. Finally, cytocompatibility study of nanoparticles was performed. Thus, we developed an effective method based on the preliminary step of protein precipitation for the formulation of PLGA nanoparticles as protein carriers for biomedical applications.

Keywords: Glycofurol (GF); Phase separation; Protein encapsulation; poly(lactic-co-glycolic acid) (PLGA); Non-toxic solvent; Nanoparticles (NPs); Lysozyme; Transforming growth factor beta1 (TGF-β1); Sustained release

Introduction

To date, protein encapsulation is a very promising area of research since many proteins are now available thanks to the recent advances in biotechnology. However, their use for therapeutic purpose remains a challenge in the field of nano and microencapsulation due to physical and chemical instability, proteolysis and short half-life. Protein encapsulation into polymeric systems such as PLGA particles has proven to be a versatile approach to protect these biomolecules, to allow their sustained delivery and to improve their therapeutic efficacy [1,2]. Therefore, various methods have been used for the formulation of PLGA particles loading proteins such as nanoprecipitation [3,4], spray-drying and spray-freeze-drying [5-7], phase separation (coacervation) [8,9], water/oil/water (w/o/w) [10,11], solid/oil/water (s/o/w) [12,13] and other derivative methods [14,15]. The main drawback of these techniques is the use of volatile organic solvents which have a potential toxicity and so are considered harmful to human health and the environment. For example, halogenated solvents such as methylene chloride and chloroform are commonly used to dissolve the PLGA in the encapsulation process [16,17]. As alternative, less-toxic solvents were used such as ethyl acetate [13,18], ethyl formate [19], methyl ethyl ketone [20] and acetone [21]. In this sense, non-volatile water-miscible solvents, which are safer than the volatile ones, were used like dimethyl sulfoxide and N-methyl pyrolidone [4]. Otherwise, supercritical fluids, especially CO_2, were proposed to completely substitute organic solvents or to minimize their use [22,23].

Hence, preparation of PLGA particles using non-toxic solvents would be of great interest to reduce the toxicity and to preserve protein integrity. Among these solvents, glycofurol (GF) and isosorbide dimethyl ether (DMI) appear to be the two safest injectable solvents, which have considerably low toxicity profiles confirmed by many scientific studies [24-29]. For this reason, they were used in many drug products for different therapeutic purposes [30-37] and for the formulation of PLGA particles as polymer solvents [38-42].

In our previous study, we have introduced the formulation of PLGA nanoparticles by a novel phase separation method called nanoprecipitation using glycofurol as polymer solvent [38]. In the present work, in addition to the encapsulation of lysozyme as a model protein, TGF-β1 was chosen to be loaded into these nanoparticles due to its important role in cell proliferation, differentiation and extracellular matrix metabolism [43]. Therefore, controlled release of TGF-β1 might be of great interest in cartilage tissue engineering [44,45]. Proteins were firstly precipitated using GF or a mixture of GF and DMI to ensure their stability and were then encapsulated into the nanoparticles. Physico-chemical properties of these nanoparticles were investigated and solvent residual content was quantified. Moreover, different techniques were performed to confirm the protein integrity and bioactivity upon the encapsulation. Besides, *in vitro* release of the proteins from PLGA nanoparticles was presented to ensure the utility of these carriers in drug sustained release. Furthermore, cytotoxicity of nanoparticles was assessed as preliminary evaluation of the cytocompatibility of these systems which will be used in cartilage tissue engineering application. This work is involved in a global project which aims to conceive novel biomaterials composed of implantable synthetic extracellular matrices combined with TGF-β1-loaded particles for cartilage regeneration. However, the application of obtained polymeric systems might be extended to any domain requiring protein encapsulation.

Materials and Methods

Materials

Lysozyme (14 kDa) from chicken egg-white, *Micrococcus*

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Received September 24, 2014; **Accepted** November 06, 2014; **Published** November 15, 2014

Citation: Swed A, Cordonnier T, Fleury F, Boury F (2014) Protein Encapsulation into PLGA Nanoparticles by a Novel Phase Separation Method Using Non-Toxic Solvents. J Nanomed Nanotechnol 5: 241. doi: 10.4172/2157-7439.1000241

lysodeikticus, glycofurol (tetraglycol or α-[(tetrahydro-2-furanyl) methyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl, isosorbide dimethyl ether (1,4:3,6-Dianhydro-2,5-di-O-methyl-D-glucitol), dimethyl sulfoxide (DMSO), NaCl, Lutrol® F68 (poloxamer 188) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Ethanol was obtained from Fischer Scientific. Human TGF-β1 was purchased from Peprotech (Paris, France). TGF-β1 ELISA Kit was obtained from R&D Systems (Lille, France). Micro-BCA protein assay reagent kit was purchased from Pierce (Bezons, France). Uncapped 75/25 PLGA provided by Phusis (Saint-Ismier, France) had a mean molecular weight of 21,000 Da (polydispersity index I=1.8) as determined by size-exclusion chromatography (standard: polystyrene). Phosphate buffered saline (PBS) was purchased from Lonza biowhittaker®, Belgium. Dulbecco's modified eagle medium (DMEM) was provided by Gibco®, Life Technology. Ultrapure water was obtained from a Milli-Q® Advantage A10 system (Millipore, Paris, France). All samples were lyophilized in Freeze-Dryer (Lyovax GT, Steris®, France) for 18 h.

Methods

Protein precipitation and encapsulation: Lysozyme precipitates, optimized by Giteau *et al.* [46], were used for the encapsulation of the protein. Precisely, 975 µl of glycofurol (GF) was added to 25 µl of 0.16 M NaCl solution containing 500 µg lysozyme to obtain a suspension of protein precipitates. TGF-β1 was precipitated in similar method but adapted to this protein. Mixture of 120 µl GF and 75 µl of isosorbide dimethyl ether (DMI) was added to 5 µl of Tris-HCl 0.05M, NaCl 2M solution (pH=7.4) containing 15% w/v Lutrol® F68 and 10 µg TGF-β1 to obtain a suspension of protein precipitates. The encapsulation of protein was then performed as previously described in our paper [38]. Briefly, 100 µl of the suspension of protein precipitates was added into 300 µl of 12% w/v PLGA solution in GF to finally obtain a suspension of protein precipitates in polymer solution. This suspension was then gently mixed with 100 µl of ethanol right before 1.5 ml of 1% Lutrol® F68 solution was added into this mixture to start the phase separation and thus to produce the nanoparticles. Thereafter, 15 ml of 6% Lutrol® F68 solution in 1.25 mM glycine buffer (solution A) was introduced into the suspension of nanoparticles. After 15 min, 25 ml of the solution A was added and the final suspension was left to stand for 16 h at room temperature (≈25°C) for the extraction step. For the preparation of blank PLGA nanoparticles, 100 µl of protein precipitates was replaced by 100 µl of GF or mixture of GF and DMI. It should be noted that the pH of the solution A is 10.35 and 9 in the case of lysozyme and TGF-β1 respectively. Finally, the suspension of nanoparticles was concentrated by centrifuging and then freeze-dried for further quantification.

Characterization of protein-loaded nanoparticles: The morphology of the nanoparticles was performed by transmission electron microscopy (TEM, JEOL, JEM1400, Japan). Drops of nanoparticle suspension diluted in ultrapure Milli-Q® water were deposited in carbon-coated copper grids and negatively stained with 1% phosphotungstic acid solution, the excess solution was blotted off using filter paper, and the grids were air dried before observation. Nanoparticle size was determined by dynamic light scattering and zeta potential was measured by electrophoretic light scattering principle using a Nanosizer® ZS (Malvern Instruments, Worcestershire, UK). Suspensions of nanoparticles diluted in ultrapure water to a suitable concentration were used for these analyses. Data analysis was performed in automatic mode at 25°C. Measured size was presented as the average value of 20 runs, with triplicate measurements within each run.

Quantification of the proteins

Quantification of the model protein (lysozyme): The total amount of each batch of freeze-dried nanoparticles was dissolved in 1 ml of DMSO (3 batches, 3 experiments per batch). After 1 h at room temperature, 3 ml of 0.01M HCl was added into the solution. The solution was left for an additional hour for protein extraction and then samples were taken for further analyses of total and bioactive lysozyme.

Total lysozyme quantification: Total lysozyme content in the nanoparticles was measured by micro-BCA protein Kit according to the manufacturer's procedure [47]. Briefly, samples were centrifuged at 10,000 g for 30 min to eliminate polymer precipitates and then 100 µl of supernatant were mixed with 100 µl of BCA reagent in a 96-well microplate (Sterilin®, Thermo-Fisher Scientific, France) and incubated at 37°C for 2 h away from light. Absorbance was finally measured at 580 nm with a plate reader (Multiskan Ascent®, Labsystems, USA). A standard curve was used to calculate the protein concentration after subtraction of the control value of blank sample prepared in the same conditions as described in the previous section.

Active lysozyme quantification: The biologically active entrapped lysozyme was determined by measuring the protein activity in the presence of its substrate, *Micrococcus lysodeikticus*. Samples were diluted to an appropriate range of concentration before being incubated with 0.015% *Micrococcus lysodeikticus* suspension in Tris-HCl (0.05M, pH 7.4) buffer solution at 37°C for 4 h. Lysozyme activity determination was based on turbidity measurement at 450 nm on a spectrophotometer (Shimadzu, Japan). Amount of active protein was calculated using a standard curve.

Quantification of the therapeutic protein (TGF-β1)

Enzyme-linked immunosorbent assay (ELISA): As previously described, the total amount of each batch of freeze-dried protein-loaded nanoparticles was dissolved in 1 ml of DMSO (3 batches, 3 experiments per batch). After one hour at room temperature, 3 ml of 10 mM citric acid was added into the solution. The solution was left for an additional hour for the protein extraction. Samples were diluted to an appropriate concentration using reagent diluent according to the manufacturer's procedure. TGF-β1 was then quantified at room temperature using the corresponding ELISA Kit. Briefly, a plate (Nunc, polylabo, Strasbourg, France) was coated with capture antibody solution. After an overnight incubation, the plate was washed with PBS buffer, 0.05% Tween 20 and then blocked by adding PBS buffer, 5% Tween 20 in each well for 1 h. After washing, aliquots of standard solution and sample solution diluted in Kit reagent diluent were added and incubated for 2 h. After washing, a detection antibody solution was added and incubated for 2 h. The solution was then rinsed and a prepared streptavidin-HRP solution was added for 20 min. Afterwards, the plate was washed and a substrate solution was added to each well for 20 min. Finally, the reaction was stopped by 2N H_2SO_4 solution and the absorbance was measured at 450 nm. The calibration curve was generated with appropriate Kit standard solution.

Bioactivity assay: In order to quantify bioactive TGF-β1 released from nanoparticles, a bioassay was performed as previously described by Tesseur *et al.* [48]. This bioassay relies on the use of mouse fibroblasts isolated from TGF-β1$^{-/-}$mice (MFB-F11) stably transfected with a reporter plasmid consisting of TGF-β responsive Smad-binding elements coupled to the secreted alkaline phosphatase (SEAP) reporter gene. Briefly, MFB-F11 fibroblasts were seeded in 96-well flat bottom plates at a density of 3×10^4 cells/well in 200 µl of DMEM with 4.5 g/L glucose and 10% fetal bovine serum (FBS). After an overnight incubation

at 37°C and 5% CO_2, cells were washed twice with PBS and 100 µl of serum-free DMEM was added to each well. After 2 h of incubation, 100 µl of the sample containing TGF-β1 released from nanoparticles was added to the wells. To determine the standard curve, serial dilutions of TGF-β1 were added to additional wells. After 24 h at 37°C and 5% CO_2, 50 µl of supernatant was collected for each well in order to measure the SEAP activity using the SEAP Reporter Gene Assay, chemiluminescent kit according to the manufacturer's procedure (Roche Applied Science, Mannheim, Germany). The chemiluminescence was measured using a plate reader (Multiskan Ascent®, Labsystems, USA).

Structural integrity of lysozyme after encapsulation: Different spectroscopic techniques were used to reveal any changes in the protein structure after encapsulation. Due to the detection limit of these methods (0.06 mg/ml), only structure integrity of lysozyme was assessed.

Circular dichroism spectroscopy: Circular dichroism (CD) spectra were recorded on a J-810 CD spectrometer (Jasco, Japan) in step mode (bandwidth: 2 nm; interval: 0.1 nm; response time: 0.125 s). The CD data were collected from 200 to 260 nm using a mini-quartz cell (Hellma, Germany) with 0.2 cm path length and averaged over 5 scans to increase the signal to noise ratio. The temperature of measurement was controlled with a Peltier-effect temperature controller, and was usually 20°C. At least three CD spectra were monitored for each sample and were scaled in units of milli-degrees (mdeg). The spectra were then averaged and smoothed using a 13-point fast fourier transform (FFT) algorithm (spectra manager software).

UV-spectroscopy in the fourth derivative mode: Baseline-corrected absorbance spectra in the range of 260-330 nm were recorded at 37°C using a V-530 Jasco UV/Vis spectrophotometer. Data acquisition was in steps of 1 nm with an acquisition time of 1s per data point. The fourth derivatives of the UV spectra were calculated with Spectra Manager analysis software (Jasco, Japan) to improve the low resolution of zero-order spectra and provide information about structural changes of proteins in the local environment of tyrosine and tryptophan residues.

Fluorescence spectroscopy: Spectra were recorded on a FP-6500 spectrofluorometer (Jasco, Japan) equipped with a Peltier temperature controller. The emission spectra were measured (bandwidth: 5 nm: response time: 0.5 s; scan rate: 100 nm/min; data pitch: 0.1 nm) in a 0.2 x 1 cm mini cell (Hellma, Germany) at 25°C. The excitation wavelength was 295 nm for selective excitation of the tryptophan residue. The spectra were measured three times from 310 to 450 nm and the data were averaged to increase the signal-to-noise ratio. All the spectra were corrected for the Raman signal and background by subtracting the spectrum of the vehicle solution.

Residual glycofurol quantification: Residual glycofurol content was analyzed in blank PLGA nanoparticles using a previously described protocol [41]. The entire batch was first dissolved in 1 ml of acetone (3 batches, 3 experiments per batch). The polymer was then precipitated by 4 ml of distilled water. Afterwards, the suspension was centrifuged at 10,000 g for 30 min, and the supernatant was collected for further analysis. Four millimeters of an ammonium cobaltothiocyanate reagent solution and 4 ml of methylene chloride were added to the sample. Thereafter, this mixture was centrifuged, and the methylene chloride phase was extracted to quantify residual glycofurol by measuring the absorbance at 620 nm on a spectrophotometer (Shimadzu, Japan).

Protein release study: Protein-loaded nanoparticle suspension of an entire batch was put inside a dialysis bag with a 1,000 kDa molecular weight cut-off (Spectrum laboratories, Inc., Rancho Dominguez, CA) and placed in a beaker containing 80 ml of 0.05 M Tris-HCl buffer, pH 7.4, 0.1% w/v BSA in the case of lysozyme and 80 ml of PBS buffer, pH 7.4, 1% w/v BSA in the case of TGF-β1. The beaker was closed and placed in a water bath at 37°C under stirring. At specific time intervals, samples from release medium were withdrawn and the whole medium was replaced with fresh one. Due to the detection limit of the quantification method, only bioactive lysozyme released from nanoparticles over 15 days was assessed. In the contrary, released TGF-β1 was quantified by ELISA and bioassay over 30 days. The experiments were made in triplicate. The ratio of cumulative release was calculated based on the amount of protein obtained from the encapsulation efficiency.

***In vitro* cytocompatibility of unloaded PLGA nanoparticles:** Cytotoxicity evaluation of unloaded nanoparticles was performed using MTS assay which measures the reduction of tetrazolium salts caused by mitochondrial dehydrogenases of viable cells to water-soluble formazan product. Briefly, NIH3T3 mouse cell line, HS68 human fibroblasts and human adipose tissue stem cells (hATSC) were seeded in 24-well plates at 37°C in a 5% CO_2 incubator with a final cell density of 15×10^3 cells/cm^2. After 24 h, the culture medium was changed with unloaded nanoparticle suspension in stabilizer solution (Lutrol® F68) after dilution with culture medium to an appropriate concentration (0.1-20 mg/ml). As a control, cells were cultured in the absence of nanoparticle suspension. The cultures were further incubated for 48 h, and then NP suspension in the culture medium was entirely removed by several PBS washes followed by the addition of MTS reagent solution to each well for 1 h. The absorbance of resulting blue formazan was measured with a plate reader at 490 nm (MultiskanAscent®, Labsystems, USA). Each experiment was realized in triplicate. The relative cell viability was calculated as the ratio of the mean of optical density obtained for the sample to that of the control. Furthermore, the viability assay was used to assess the cytotoxicity of the stabilizer used for the preparation of nanoparticles suspension. Stabilizer solutions in cell culture medium were appropriately diluted to a concentration corresponding to the amount present in the Nanoparticle suspension. Values are expressed in mean ± standard deviation (SD). The differences between treated and untreated cells were evaluated using one-way ANOVA test.

Results and Discussion

Encapsulation of the proteins into PLGA nanoparticles

Many studies showed that protein stability in contact with organic solvents is enhanced when the protein is in a solid-state because of the decrease in its conformational mobility in the absence of water [3,46]. Therefore, in this paper proteins were first precipitated to ensure their stability using non-toxic injectable solvents GF and DMI [38]. The protein precipitates were then encapsulated within PLGA nanoparticles by a phase separation method called nanoprecipitation. During the encapsulation step, the pH of dispersing phase was buffered to be near to the protein isoelectric point (Pi). In our previous study, we have demonstrated the impact of aqueous phase pH on the protein encapsulation efficiency [38]. Govender *et al.* also reported the influence of aqueous phase pH on the drug entrapment [49]. It is hypothesized that the protein is less ionized at pH near its Pi and therefore less soluble in the aqueous phase than in the organic phase. This may reduce the protein leakage into the aqueous phase and thus increase the encapsulation efficiency. The mean encapsulation efficiency was 76 ± 5%; 73 ± 6% for total and active lysozyme respectively. These results emphasize high preserved activity (96%) of the total amount of lysozyme encapsulated by our process. In the case of the therapeutic

Batch	EE ± SD (%)	Size ± SD (nm)	PI ± SD	Zeta potential ± SD (mV)
Lysozyme-loaded NPs	76 ± 5	381 ± 68	0.14 ± 0.05	-29 ± 2.6
TGF-β1-loaded NPs	50 ± 7	345 ± 35	0.13 ± 0.03	-28 ± 1.3

*EE: encapsulation efficiency; SD: standard deviation; PI: mean polydispersity index expressed using a 0-1 scale (n=3)

Table 1: Characterization of PLGA nanoparticles prepared by the phase separation method*.

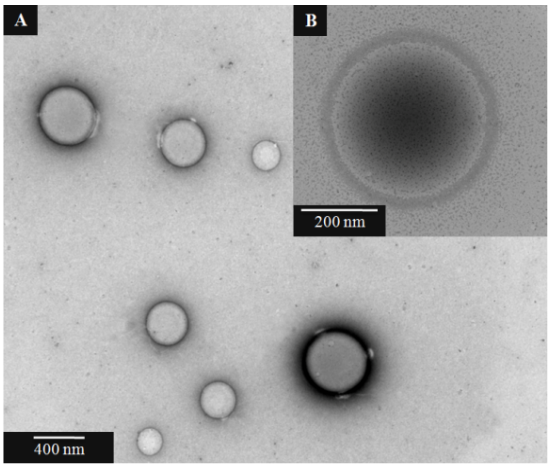

Figure 1: TEM images of nanoparticles (A), and at high magnification (B).

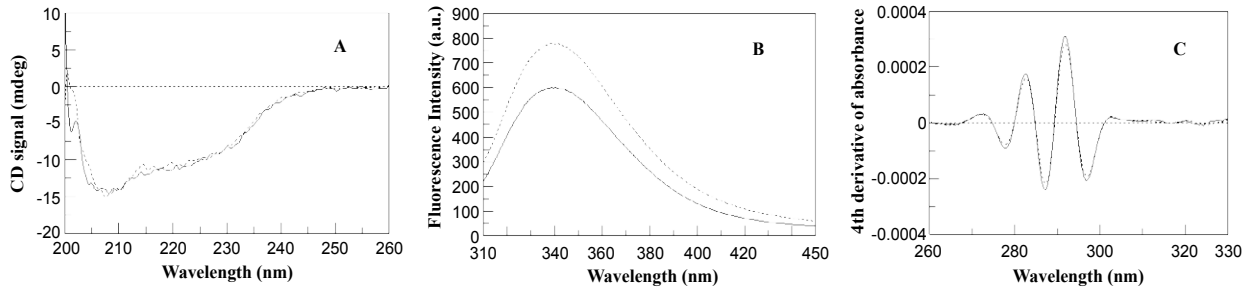

Figure 2: Results of spectroscopic studies of native lysozyme (solid curve) and encapsulated lysozyme (dotted curve).The far-UV CD, fluorescence and fourth derivative of UV spectra are shown in panel A, B and C respectively.

protein (TGF-β1) the encapsulation efficiency determined by ELISA was 50 ± 7% (Table 1).

Characterization of protein-loaded nanoparticles

The results of the TEM observation showed that the prepared nanoparticles were spherical in shape and of submicron size (Figure 1). This was further confirmed by nanoparticle size analysis using Nanosizer, which showed that the average size is about 345-381 nm with satisfactory polydispersity index (Table 1). These results are in agreement with those obtained by Bilati *et al.* in which non-volatile water-miscible solvents like DMSO were used to dissolve PLGA. Nanoparticles were also formed by a nanoprecipitation process when polymer solution was mixed with a non-solvent such as water, propanol or ethanol [4]. Zeta potential measurements showed that PLGA nanoparticles had a negative surface charge of about -28 mV (Table 1) which could be attributed to the presence of terminal carboxylic groups of the polymer on the nanoparticle surface. Comparable results were reported in several other studies [50,51].

Lysozyme structural integrity study

Different spectroscopic methods were performed to assess the structural integrity of the lysozyme after encapsulation. The structure of released lysozyme from the nanoparticles was compared to that of the native protein prepared in the same conditions (free protein as reference). Generally, if a protein becomes denatured during the encapsulation step, it will remain in the same state during the release step. Therefore, samples of lysozyme released within 24 h from nanoparticles into a PBS solution containing 0.1% w/v Lutrol F68® (pH 7.4, 37°C) was collected and concentrated using Amicon® ultra centrifugal filters (Ultracel®-3 KDa, Germany) to obtain the concentration of 0.06 mg/ml. Secondary structure of each protein was analyzed by CD measurement. The comparison between released and free lysozyme CD spectra was performed. The Figure 2A showed that the secondary structure of both proteins was unchanged and thus preserved upon and after encapsulation.

Fluorescence spectroscopy spectra showed a reduced level of fluorescence intensity without change in peak maximum which is possibly due to a quenching of the fluorescence rather than changes in the protein integrity (Figure 2B). These results were confirmed with the fourth derivative UV-visible spectra which did not show any shift of maxima in the area corresponding to tyrosine and tryptophan residues

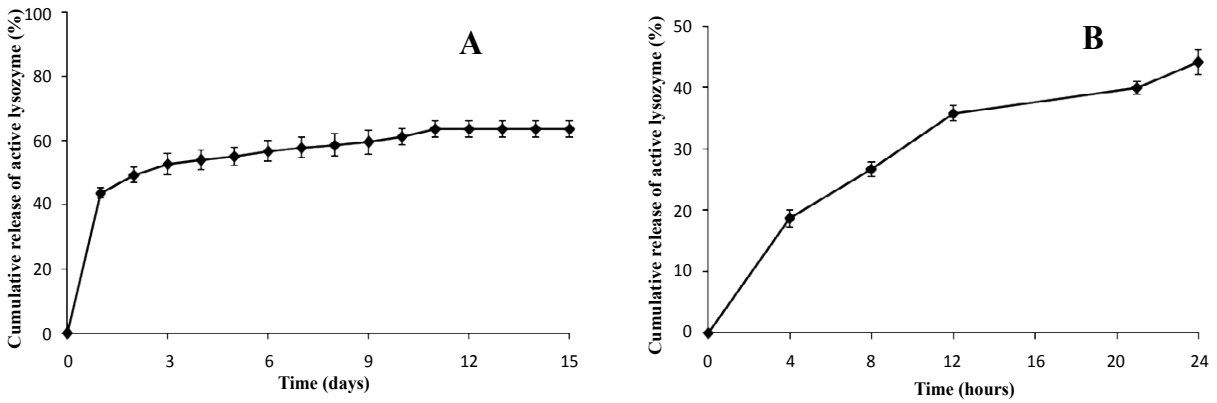

Figure 3: *In vitro* release profile of active lysozyme during 15 days (A), and during the first 24 h (B).

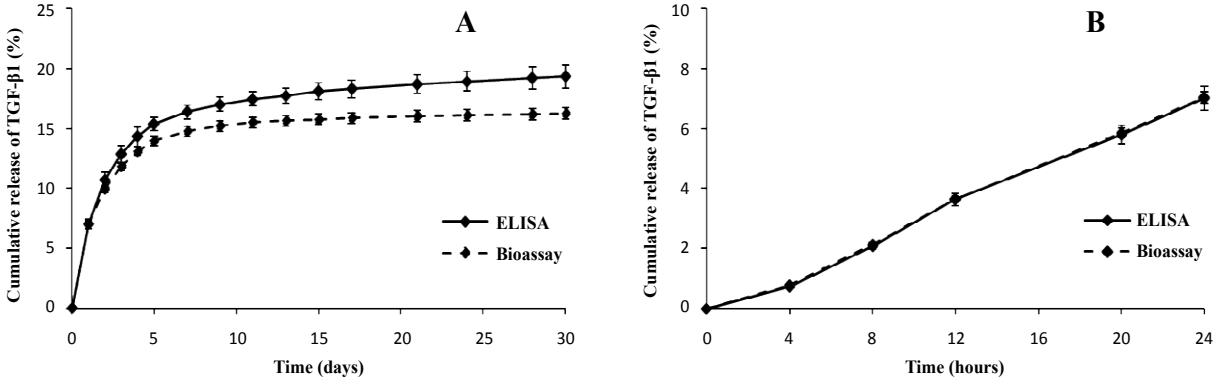

Figure 4: *In vitro* release profile of TGF-β1 during 30 days (A), and during the first 24 h (B).

(284 and 290 nm respectively) indicating no change in the secondary structure after encapsulation (Figure 2C).

Residual glycofurol content

Residual glycofurol content is of 14.2 ± 1.3 mg per 100 mg nanoparticles. In our previous paper [38], the presence of residual glycofurol was assessed using DSC analysis (Differential scanning calorimetry) by the decrease of PLGA transition temperatures from around 45°C to around 27.5°C. Comparable results were found by Allhenn *et al.* [41]. Non-volatile solvents are generally more difficult to extract compared to volatile ones. In spite of the incomplete solvent extraction, no loss of protein integrity or bioactivity was detected as described above. Furthermore, lysozyme stability was tested in glycofurol at 24°C by Aubert-Pougëssel *et al.* and results showed that 100% of biologically active protein was recovered [52].

In vitro release study of the protein from nanoparticles

The release profiles of lysozyme and TGF-β1 from nanoparticles are shown in Figures 3A and 4A. Both proteins were released in a biphasic mode. The first phase is characterized by a fast release which probably results from the solubilization of protein that usually exists near the surface. The second phase is characterized by a slow release which could be attributed to the degradation of polymer matrix leading to the diffusion of the entrapped protein. Similar profiles of protein release are found in the literature for such nano-sized systems [53-55].

The extents of lysozyme and TGF-β1, released in the first day, were about 44% and 7% respectively (Figures 3B and 4B). These results are believed to be related to the fact that lysozyme has a higher quantity of loaded protein than TGF-β1 (50 µg and 5 µg respectively for 36 mg of PLGA theoretically) and thus a higher initial release. Probably, with the increase in drug loading, a large protein gradient is formed within particles toward release medium which induces a faster drug release and consequently a higher burst effect [56]. On the other hand, the release rate of lysozyme from nanoparticles was higher than that of TGF-β1 over 15 days (64% and 18% respectively, Figures 3A and 4A), which indicates that release kinetics of the proteins from nanoparticles are influenced by the physicochemical characteristic of each protein (Lysozyme: pi=11, MW=14 kDa, TGF-β1: pi=8.6, MW=25 kDa). Due to its higher molecular weight, TGF-β1 has likely less diffusion rate and more interaction with the polymer matrix than lysozyme and thus a lower release rate. Interestingly, the amount of total TGF-β1 released after 24 h quantified by ELISA is equal to that of active protein quantified by bioassay (Figure 4B). This result confirms that the biological activity of TGF-β1 was preserved after encapsulation. Similar results were also obtained for lysozyme as previously described indicating the versatility of our method for protein encapsulation. After 24 h of release study, PLGA nanoparticles were recovered and freeze-dried to quantify the remaining active lysozyme. 56% of active lysozyme was found to be remaining in each sample. While the released active lysozyme added to the remaining active protein is equal to 100% after 24 h, it is not the case after 15 days where about 12% of active lysozyme was found

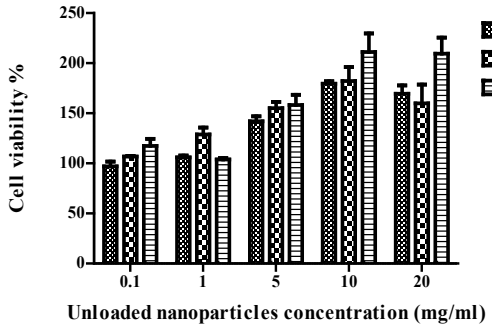

Figure 5: Effect of unloaded PLGA nanoparticles on percentage viability of NIH3T3, HS68 and hATSC cells after 48 h of incubation as shown by a MTS assay.

to be remaining inside the nanoparticles, which means that 24% of the total amount of active lysozyme was denatured. Similar results were observed for TGF-β1 quantified by ELISA. After 24 h, the total amount of TGF-β1 (released amount added to remaining amount) is equal to 100% whilst after 15 days, about 36% of the total amount was denatured. Thus, we believe that protein denaturation occurs inside the nanoparticles during the release step since we have proven in a preliminary study that both proteins are stable in the release medium. However, over the whole time of release study, bioactive released TGF-β1 quantified by bioassay represented 84% of the total amount of released TGF-β1 quantified by ELISA which is also supposed to be related to the protein denaturation inside the nanoparticles during the release step. It should be noted that Lutrol® F68 was used during the precipitation step in the case of TGF-β1 to enhance its stability in the polymer matrix and thus improving its release as described in many studies [57,58]. However, an incomplete release was observed for each protein. These findings was reported earlier in the literature [59,60] and could be prone to many denaturation mechanisms such as non-specific adsorption onto the polymer, acid-induced aggregation due to polymer degradation, and moisture-induced aggregation [61,62]. Many strategies have been proposed by different authors to improve protein stability within PLGA particles during the release step [63,64]. In order to minimize protein adsorption and to enhance water uptake and thereby promoting the release rate, pegylated PLGA or a blend of PEG and PLGA were used [16,65]. Basic salts such as zinc carbonate and magnesium compounds were proposed to inhibit acid-induced protein degradation inside the polymeric particles during the release [66,67]. Other additives like ammonium sulphate were also used to minimize moisture-induced protein aggregation during slow PLGA particles hydration [68]. These approaches are now under investigation and would be considered in a future work.

Cytocompatibility study

The safety of PLGA particles as drug delivery systems is very important for medical applications. However, PLGA nanoparticles are well known to be biodegradable and biocompatible *in vitro* and *in vivo* compared to other nanoparticles of a similar size range such as zinc oxide, ferrous oxide and fumed silica which exhibit toxic effects when applied in the field of nanomedicine [69,70].

An *in vitro* cytotoxicity study was conducted to assess the cell viability following exposure to the unloaded PLGA nanoparticles, NIH-3T3, HS68 and hATSC cells were cultured in the presence of unloaded PLGA nanoparticles. MTS assay showed excellent cell viability even at high nanoparticles concentration (20 mg/ml) (Figure 5). Furthermore, the cells viability in NP suspension was comparable to that in stabilizer solution (Lutrol® F68) at a concentration corresponding to the amount present in the nanoparticle suspension (data not shown). These results confirm the cytocompatibility of PLGA nanoparticles which are in agreement with data obtained earlier in the literature [71-73]. It should be noted that the proliferation of cells was observed after 48 h of incubation with unloaded NPs starting from concentration of 5 mg/ml. Various parameters might affect the cell proliferation such as the methodology of NP formulation, residual solvent and cell lines type. However, more specific assays on cell proliferation, such as assays based on DNA quantification, should be considered in future work in order to confirm this effect. In this experiment, our goal was to assess the cytocompatibility of obtained nanoparticles which will be used for tissue engineering applications where the effect on cell proliferation might be of interest.

Conclusion

In this study, protein-loaded nanoparticles were prepared using non-toxic solvents by a novel phase separation method based on a preliminary step of protein precipitation. Interestingly, structural integrity and biological activity of the encapsulated proteins were preserved and good encapsulation efficiencies were also achieved. Sustained released of the proteins from the prepared nanoparticles was assessed. Nevertheless, the optimization of protein release is still in progress. This work can serve as an alternative for classic encapsulation methods which use volatile toxic solvents. Further efforts will be provided for the development of the final product in the form of hybrid biomaterials for regenerative medicine application. Therefore, the potential of these polymeric systems to allow proliferation and differentiation of chondrocytes *in vitro* will be firstly assessed in a future work. Then, TGF-β1-loaded PLGA nanoparticles could be combined to a hydrogel containing adult stem cells as an implantable synthetic extracellular matrix for cartilage regeneration. In addition to the interest of obtained nanoparticles for tissue engineering, their application can be extended to any domain requiring protein encapsulation.

Acknowledgment

Acknowledgments are made to the SCIAM ("Service Commun d'Imagerie et d'Analyse Microscopique") for transmission electron microscope images. We thank the "Région Pays de la Loire" (Bioregos 2 project), ANR ("Agence Nationale pour la Recherche", grant (ANR-09-PIRI-0004-01), the French and Syrian education ministers for the financial support.

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Citation: Swed A, Cordonnier T, Fleury F, Boury F (2014) Protein Encapsulation into PLGA Nanoparticles by a Novel Phase Separation Method Using Non-Toxic Solvents. J Nanomed Nanotechnol 5: 241. doi: 10.4172/2157-7439.1000241