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Research paper

Development of a non-toxic and non-denaturing formulation process for encapsulation of SDF-1α into PLGA/PEG-PLGA nanoparticles to achieve sustained release

Muhammad Haji Mansor[a,b], Mathie Najberg[a,c], Aurélien Contini[a], Carmen Alvarez-Lorenzo[c], Emmanuel Garcion[a,1], Christine Jérôme[b,1], Frank Boury[a,*]

[a] CRCINA, INSERM, Université de Nantes, Université d'Angers, Angers, France
[b] Center for Education and Research on Macromolecules (CERM), Université de Liège, Liège, Belgium
[c] Departamento de Farmacologia, Farmacia y Tecnología Farmacéutica, R & D Pharma Group, Facultad de Farmacia, Universidade de Santiago de Compostela, Santiago de Compostela, Spain

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ABSTRACT

Chemokines are known to stimulate directed migration of cancer cells. Therefore, the strategy involving gradual chemokine release from polymeric vehicles for trapping cancer cells is of interest. In this work, the chemokine stromal cell-derived factor-1α (SDF-1α) was encapsulated into nanoparticles composed of poly-(lactic-co-glycolic acid) (PLGA) and a polyethylene glycol (PEG)-PLGA co-polymer to achieve sustained release. SDF-1α, and lysozyme as a model protein, were firstly precipitated to promote their stability upon encapsulation. A novel phase separation method utilising a non-toxic solvent in the form of isosorbide dimethyl ether was developed for the individual encapsulation of SDF-1α and lysozyme precipitates. Uniform nanoparticles of 200–250 nm in size with spherical morphologies were successfully synthesised under mild formulation conditions and conveniently freeze-dried in the presence of hydroxypropyl-β-cyclodextrin as a stabiliser. The effect of PLGA carboxylic acid terminal capping on protein encapsulation efficiency and release rate was also explored. Following optimisation, sustained release of SDF-1α was achieved over a period of 72 h. Importantly, the novel encapsulation process was found to induce negligible protein denaturation. The obtained SDF-1α nanocarriers may be subsequently incorporated within a hydrogel or other scaffolds to establish a chemokine concentration gradient for the trapping of glioblastoma cells.

1. Introduction

Stromal cell-derived factor-1α (SDF-1α) is a chemokine composed of 68 amino acids [1] that binds to its cognate receptor, C-X-C chemokine receptor type 4 (CXCR4) [2]. One of its important physiological functions is to retain high concentrations of CXCR4-expressing stem and progenitor cells within the bone marrow by creating a positive concentration gradient from the blood to this organ [3]. In the events of tissue damage, the SDF-1α expression at the injury site is elevated [4–6] in a simultaneous fashion to the increased SDF-1α degradation in the bone marrow [7,8] to allow mobilisation of the stem and progenitor cells and their subsequent chemoattraction to the site of damage. In addition to its roles in tissue repair and regeneration, SDF-1α-mediated chemotaxis is also implicated in tumour metastases. CXCR4-expressing cancerous cells that are present in the blood or lymphatic circulation after getting dislodged from the primary tumour site can be

Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; CXCR4, C-X-C chemokine receptor type 4; DL, drug loading; DMEM, Dulbecco's Modified Eagle's Medium; DMI, isosorbide dimethyl ether/dimethyl isosorbide; DMSO, dimethyl sulfoxide; EE, encapsulation efficiency; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; GBM, glioblastoma; HCl, hydrochloric acid; HPBCD, hydroxypropyl-β-cyclodextrin; ICH, International Conference on Harmonization; LNC, lipid nanocapsules; Mn, number-average molecular weight; Mw, weight-average molecular weight; NaCl, sodium chloride; NIH3T3, mouse fibroblast cell line NIH3T3; NMR, nuclear magnetic resonance; P188, poloxamer 188; PBS, phosphate-buffered saline; PDI, polydispersity index; PE, precipitation efficiency; PEG, polyethylene glycol; pI, isoelectric point; PLGA, poly-lactic-co- glycolic acid PLGA-COOH, poly-lactic-co- glycolic acid with uncapped carboxylic acid terminals; PLGA-COOR, poly-lactic-co- glycolic acid with capped carboxylic acid terminals; PS, polystyrene; PVA, polyvinyl alcohol; SD, standard deviation; SDF-1α, stromal cell-derived factor-1α; SEC, size exclusion chromatography; SEM, scanning electron microscopy; T_c, collapse temperature; TEM, transmission electron microscopy; Tris, tris(hydroxymethyl)-aminomethane; U87-MG, human malignant glioblastoma cell line U87-MG

* Corresponding author at: Cancer and Immunology Research Centre Nantes-Angers (CRCINA), INSERM U1232, Team GLIAD, Université d'Angers, IBS – CHU Angers, 4 rue Larrey, 49933 ANGERS CEDEX 9, France.
E-mail address: frank.boury@univ-angers.fr (F. Boury).
[1] Equivalent contribution.

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chemoattracted to SDF-1α-secreting sites such as the bone marrow [9], liver [10] and lymph nodes [11] for future metastatic growth. This pathological role of SDF-1α has inspired the design of implants capable of creating a SDF-1α concentration gradient for trapping CXCR4-expressing cancerous cells relevant to multiple types of malignant cancers such as glioblastoma (GBM) [12], gastric carcinoma [13] and small-cell lung cancer [14].

Due to its solubility and rapid diffusion in physiological media, a sustained delivery of SDF-1α is a prerequisite for establishing its concentration gradient. Encapsulation of SDF-1α into polymeric nanoparticles is a credible strategy for achieving a gradual SDF-1α release at the site of application. In this regard, poly-(lactic-co-glycolic acid) (PLGA) is a polymer of choice for nanoparticle formulations, owing to its biocompatibility, biodegradability and most importantly, its status as a Food and Drug Administration-approved pharmaceutical excipient [15]. However, due to its hydrophobicity, the formation of stable PLGA nanoparticles often necessitates the use of amphiphilic surfactants such as polyvinyl alcohol (PVA) [16,17] and poloxamer 188 (P188) [18] in the formulation process. Although these surfactants are innocuous when used in isolation, residual PVA and P188 bound to the PLGA nanoparticle surfaces have been reported to induce toxicities especially at nanoparticle concentrations exceeding 1 mg/mL [19], which are relevant to many local applications of PLGA-based nanoparticles. The development of a PLGA-based nanoparticle formulation process that avoids or reduces the need for surfactants is therefore in demand.

To encapsulate hydrophilic drugs in hydrophobic PLGA matrices, the double emulsion (water/oil/water) process is often preferred [20,21]. While this process is excellent for encapsulating small hydrophilic molecules, problems can arise with drugs of complex structures such as proteins. The first step of this process that involves emulsification of a protein solution in the polymer-containing organic phase can lead to adsorption of protein molecules to the water/organic solvent interface and their subsequent unfolding. The structural instability of dissolved proteins is actually exaggerated by their conformational flexibility that makes it possible for their hydrophobic pockets to be externalised to make contact with the organic phase upon emulsification [22]. Thus, a possible solution to promote protein stability during encapsulation is by minimising their conformational mobility through the use of proteins in solid form. In this regard, techniques such as freeze-drying and spray-freeze-drying have been employed to produce fine protein particles for subsequent encapsulation [23,24]. However, these techniques themselves can induce substantial protein structural changes. On the other hand, proteins in solution can be precipitated by adding a water-miscible organic solvent [25]. This technique produces homogenous nano-sized protein particles without affecting protein structures and bioactivities, and therefore serves as a suitable protein treatment prior to encapsulation.

Currently, the encapsulation of proteins or peptides into PLGA nanoparticles typically involves the use of toxic halogenated solvents such as chloroform and dichloromethane as the polymer solvent [26–28]. Other common harmful PLGA solvents include acetonitrile [29], N-methylpyrrolidone [30], N,N-dimethylformamide and tetrahydrofuran [31]. These solvents belong to Class 2 according to the International Conference on Harmonization (ICH), which are harmful solvents that can pose serious threats to patient safety [32]. Less toxic solvents such as acetone [33], ethyl acetate [34] and dimethyl sulfoxide [30] are being increasingly used as alternatives. Nevertheless, they are still regarded as potential hazards to human health by the ICH. Differently, the safety of non-volatile water-miscible organic solvents such as glycofurol and isosorbide dimethyl ether (DMI) have been demonstrated *in vivo*. They have been recommended as solvents suitable for intravascular injections [35,36] due to their negligible toxicity. Thus, the use of these solvents for protein encapsulation into PLGA-based nanoparticles is well-motivated.

In the present study, an amphiphilic polyethylene glycol (PEG)-PLGA co-polymer was synthesised and used together with hydrophobic PLGA polymers to produce stable nanoparticles via a phase separation method without the use of conventional surfactants. In addition, the non-toxic DMI was utilised as a solvent for the PLGA polymers and the PEG-PLGA co-polymer. To the best of our knowledge, this is the first example of the use of this benign solvent to produce PLGA/PEG-PLGA nanoparticles. PLGA with capped or uncapped carboxylic acid terminals were combined with the PEG-PLGA co-polymer at different proportions to produce nanoparticles of different size distributions and surface charges. The nanoparticles were then freeze-dried in the presence of three excipients to explore the possibility of obtaining nanocarriers with a prolonged shelf-life. Following the optimisation of the PLGA/PEG-PLGA nanoparticle synthesis, lysozyme (14.3 kDa, isoelectric point: 11.35) was initially used as a model protein to optimise the encapsulation of SDF-1α (8.0 kDa, isoelectric point: 10.5). To preserve the protein bioactivity throughout the formulation process, lysozyme and SDF-1α precipitates were prepared by mixing respective protein solutions with glycofurol prior to encapsulation. Then, *in vitro* release of lysozyme and SDF-1α from the PLGA/PEG-PLGA nanoparticles was studied. The bioactivity of the released SDF-1α was subsequently assessed in terms of its capacity to induce migration of CXCR4-expressing human GBM cells (U87-MG). Finally, the cytocompatibility of the newly-developed nanoparticles was assessed *in vitro*.

2. Materials and methods

2.1. Materials

PLGA with capped carboxylic acid terminals and PEG-PLGA co-polymer were synthesised as described in Section 2.2. PLGA 75:25 with uncapped terminals (Resomer® RG752H, Mw = 9850 Da, polydispersity index (PDI) = 2.4), lysozyme of chicken egg white, *Micrococcus lysodeikticus*, glycofurol (tetraglycol or tetrahydrofurfuryl alcohol polyethyleneglycol ether), isosorbide dimethyl ether (dimethyl isosorbide), dimethyl sulfoxide (DMSO), sodium chloride, poloxamer 188 (Lutrol® F68), glycine, sucrose, trehalose, 37% hydrochloric acid, 10 M sodium hydroxide, tris(hydroxymethyl)aminomethane (Tris) base (Trizma®) and agarose with low gelling temperature were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). DL-lactide (Purasorb® DL) and glycolide (Purasorb® G) were obtained from Purac Biomaterials, Frankfurt, Germany. Bovine serum albumin fraction V was obtained from Roche Diagnostics (Mannheim, Germany), human SDF-1α from Miltenyi Biotech (Paris, France), hydroxypropyl-β-cyclodextrin (Kleptose® HPBCD) from Roquette (Lestrem, France), Dulbecco's phosphate-buffered saline (Biowhittaker®) from Lonza (Verviers, Belgium), and Dulbecco's Modified Eagle's Medium (Gibco® DMEM) from Thermo Fisher Scientific (Villebon sur Yvette, France). Ultrapure water dispensed from a Milli-Q® Advantage A10 system (Millipore, Paris, France) was used in all experiments.

2.2. Synthesis and characterization of PLGA with capped carboxylic acid terminals (PLGA-COOR) and PEG-PLGA co-polymer

2.2.1. Synthesis

The synthesis of PLGA-COOR was adapted from the ring-opening polymerization method described by Yoo and Park [37]. Briefly, a mixture of DL-lactide (Purasorb® DL) and glycolide (Purasorb® G) in the molar ratio of 3:1 was heated with the initiator benzyl alcohol to 140 °C under nitrogen atmosphere for complete melting. The use of this initiator would result in a benzyl group being the R-group in the PLGA-COOR product. Then, 0.04% (w/w) stannous octoate was added, and the reaction mixture was further heated to 180 °C. The temperature was maintained for 3 h for polymerization to take place under static vacuum. The polymer was then recovered by dissolution in dichloromethane before precipitation in heptane. The precipitate was subsequently filtered and dried at 25 °C for 24 h under vacuum. For the synthesis of PEG-PLGA co-polymer, the same procedure was adopted