Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics

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Formulating protein therapeutics into nanoparticles (NPs) of poly(lactic-co-glycolic acid) (PLGA) provides key features such as protection against clearance, sustained release and less side effects by possible attachment of targeting ligands. These NPs also offer the potential for protein combination therapy, which is expected to exploit synergetic bioresponses, avoid multiple dosage regimens and consequent mis-dosing. Since the conventional manufacture of protein-loaded PLGA NPs is still associated with low-throughput, new continuous manufacturing methods such as microfluidics have been established. Herein, PLGA NPs continuously manufactured through microfluidics are proposed for co-formulation and -delivery of two model proteins consisting of bovine serum albumin (BSA) conjugated to either fluorescein (FITC) or tetramethylrhodamine (TRITC) isothiocyanates. Protein co-formulated NPs of 100 nm, monodispersed and with 70% of association efficiency were obtained. The microfluidic setup allowed a production rate of around 7 g of particles per day and demonstrated scale-up capacity. Model proteins were released in a controlled manner and without significant changes in their secondary structure. Studies in macrophage-like cells proved that protein co-formulated PLGA NPs did not impair metabolic activity (>70%). The cellular association of the proteins was around 2-times higher when co-formulated into PLGA NPs, compared to the free protein controls. Moreover, the cellular association of the co-formulated proteins was 4-times higher than the physical mixture of NPs individually loaded with each protein type. This work has demonstrated the effectiveness of continuously manufactured PLGA NPs for co-formulating and enhancing the cellular association of co-delivered model proteins, providing a proof-of-concept foundation for future protein combination nanotherapies.

1. Introduction

The protein therapeutic market is an important segment of the pharmaceutical industry and is anticipated to be worth $230 billion by 2021. ¹ The exponential growth of this market is correlated with the superior therapeutic performance over small-molecule drugs, which is due to higher bioactivity and specificity.² Protein-based therapeutics have therefore been widely investigated for the treatment of various diseases, with more than 200 Food and Drug Administration (FDA)-approvals for clinical use since 1980.³

Although proteins hold great potential as therapeutics, their delivery poses a significant challenge. Protein therapeutics are highly affected by physiological conditions, which may compromise their stability and trigger their proteolytic degradation, opsonization, as well as complexation with biologic products leading to conformational changes and consequent loss of function.⁴ Moreover, lipophilic cell membranes restrict the passage of proteins with intracellular targets, which are subject to challenges in interacting with cells and accessing sub-cellular compartments.⁵ As a result, protein therapeutics can be diverted away from their target, resulting in side effects emerging from off-site accumulation and the need for repeated administrations. Polymer-based nanoparticles (NPs) have been identified as a promising strategy to circumvent these drawbacks by offering a protective vehicle for the formulation of protein therapeutics. This is

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because, they have been found to prevent the rapid clearance of the protein therapeutics whilst providing their controlled release over time.6,7 The FDA-approved poly(lactic-co-glycolic acid) (PLGA) polymer is the most widely utilized polymer for protein therapeutic-loaded NPs with biodegradable and biocompatible properties.8 PLGA is a surface-functionalizable polymer that allows further attachment of a variety of targeting ligands, which are key to inducing higher accumulation of the NPs at the target site, therefore reducing potential side effects.9 However, conventional bulk processes for synthesis of protein therapeutic-loaded PLGA NPs are often related to slow- and low-throughput, unsuccessful association efficiency and lack of batch-to-batch consistency.10,11 These pitfalls are being overcome by the rapid development of continuous manufacturing methods such as microfluidics that offer the possibility of precisely control the particle-formation environment and enable the production of PLGA NPs with tunable physicochemical characteristics and higher drug loading capacity, as well as monodisperse batches.12–14 Microfluidics also save reproducible, time-saving up-scale manufacturing of NPs, which is crucial to reach industrial and clinical batch sizes.15

Another challenge with manufacturing protein therapeutic-loaded PLGA NPs is the harsh environment encountered when employing the double emulsion systems commonly used in the formulation process. The shear stress inherent to the immiscibility of the phases involved in these systems leads to the interfacial adsorption of proteins, which is the major cause of their denaturation.16,17 Typical nanoprecipitation-based processes would be more appropriate due to the absence of oily-aqueous interfaces, although they are mostly designed for the formulation of highly hydrophobic cargos.18 Some authors have introduced modifications in the nanoprecipitation process to accomplish the formulation of hydrophilic compounds into hydrophobic polymeric matrices, however, these modifications generally require more complex co-solvent systems.19–24

The development of PLGA NPs for protein delivery has made notable advances, while their potential to co-formulate protein therapeutics and perform combination therapy is yet to be fully investigated. Co-formulation of multiple therapeutics into polymeric NPs is expected to simplify delivery to patients by reducing multiple dosage regimens, achieve synergetic bioresponses by tuning the ratio of the loaded cargos, as well as to provide a simultaneous targeting to multiple therapeautical active sites.25 Combination therapy may be particularly useful for multi-cytokine treatments, since immune responses trigger several biochemical cascades in which many molecular networks need to be targeted simultaneously.26

Bovine serum albumin (BSA) is an ideal protein candidate for preliminary model studies due to its relatively high molecular weight (around 66.5 kDa) and economical price point.27 The conjugation of fluorochromes as either fluorescein (FITC) or tetramethylrhodamine (TRITC) isocyanates with BSA is well known to significantly change its physicochemical properties, which produces fluorochrome-dependent protein profiles.28 For example, protein solubility can be considerably altered by the labelling fluorochrome, due to a net-increase in the total number of attractive sites in the protein-fluorochrome system, as reported by Quinn et al.29 Therefore, BSA-FITC and BSA-TRITC can be distinguished from each other and from the unlabelled BSA with respect to their physicochemical characteristics and used as two different model proteins, to study continuous manufacturing and encapsulation into polymeric NPs.

This study reports PLGA NPs for all-in-one co-formulation and -delivery of two models of protein therapeutics consisting of BSA-FITC and BSA-TRITC. The study addresses continuous manufacturing by investigating a microfluidic modified-nanoprecipitation technique for the production of protein co-formulated PLGA NPs, with further assessment of their impact in the association of the cargos with macrophage-like cells.

2. Experimental

2.1 Materials

The X-junction microfluidic device (Quartz Droplet Junction Chip, 190 μm etch depth) was acquired from Dolomite (Royston, UK), BSA-FITC, BSA-TRITC, Tween®-80, sodium chloride, phosphate buffered saline (PBS), phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA); acetonitrile from Thermo Fisher Scientific (Waltham, MA, USA); high glucose with ultraglutamine Dulbecco's modified Eagle medium (DMEM) from Lonza (Basel, Switzerland); Triton X-100 from Spī-Chem (West Chester, PA, USA); and fetal bovine serum (FBS) and penicillin-streptomycin from Gibco (Waltham, MA, USA). Acid-terminated PLGA polymer (50 : 50 lactic to glycolic ratio, molecular weight of 10 kDa) was kindly provided by Dr Mohammad Al-Natour and its synthesis was described elsewhere.30

The human monocytic leukaemia THP-1 cell line was purchased from ATCC (Manassas, VA, USA).

2.2 Methods

2.2.1 Nanoparticles manufacturing and process optimization. The organic phase used for the microfluidic manufacturing of the NPs consisted of a mixture of PLGA in acetone, for which the concentration was optimized between 0.25% and 1% w/v. The aqueous phase, in turn, was kept as a 2% w/v solution of Tween®-80, and both BSA-FITC and BSA-TRITC were added to this phase to obtain protein-loaded PLGA NPs. For the BSA-FITC and BSA-TRITC individual formulation, a concentration of 33 μg mL⁻¹ and 67 μg mL⁻¹ of BSA-FITC and BSA-TRITC in the 2% w/v solution of Tween®-80, respectively, was used. Whereas, for the BSA-FITC and BSA-TRITC co-formulation, a concentration of 25 μg mL⁻¹ and 8 μg mL⁻¹ of BSA-FITC and BA-TRITC in the 2% w/v solution of Tween®-80, respectively, was used.

The microfluidic chip displayed a three-inlets X-layout, in which the organic and aqueous phases were injected through the central and the two outer inlets, respectively (Fig. 1).
total flow rate was varied from 25:75 to 500:1500 μL min⁻¹ regarding the organic : aqueous phases. Each experiment was conducted until 10 mg of PLGA NPs were produced (equivalent to 2 min run time using the final flow rate conditions). NPs were washed three times with ultrapure water and recovered by ultrafiltration using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Billerica, MA, USA) with a molecular weight cut-off (MWCO) of 100 kDa.

**Scale-up experiment.** A scale-up experiment was performed in relation to the mass of PLGA in the final batch of unloaded NPs. The batch size was scaled 5- and 25-times, corresponding to a final mass of PLGA of 50 mg and 250 mg, respectively.

### 2.2.2 Characterization of nanoparticles

**Mean particle size, size distribution and surface charge.** NPs were characterized for their average size (Z-average) and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta-potential (ζ-potential) through laser Doppler anemometry (LDA), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). For these measurements, samples were diluted (1:100) in an ionic solution of 10 mM sodium chloride.

**Association efficiency.** The amount of loaded BSA-FITC and BSA-TRITC was directly quantified in order to determine the association efficiency (AE) of the developed nanosystems. The calculation of AE followed eqn (1).³¹

\[
AE_{\text{BSA}}(\%) = \frac{\text{Quantified loaded mass of BSA}}{\text{Initial mass of BSA}} \times 100 \tag{1}
\]

The amount of loaded BSA-FITC and BSA-TRITC was spectrophotometrically quantified after the extraction of the proteins from the matrix of the NPs according to a protocol adapted from.³² Briefly, the protein-loaded NPs were treated with a solution of 3 M NaOH for 24 h at 100 rpm in an orbital shaker (Thermo Scientific, Madison, WI, USA), to promote the disaggregation of the polymeric matrix and release of the associated BSA-FITC and BSA-TRITC. The suspension was then sonicated for 1 h and centrifuged at 14 000 rpm for 15 min (Hermle Z 300, Hermle Labortechnik GMBH, Wehingen, DE). The supernatant was filtered through a 0.22 μm syringe filter (Milllex GP, Millipore, Bedford, MA, USA). The absorbance of the filtrate was read in a Varian Cary® 50 Bio UV-VIS spectrophotometer (Agilent Technologies, Stockport, UK) at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

**Morphology.** The morphological features of the NPs were analyzed by transmission electron microscopy (TEM) using a Philips Tecnai BioTwin-12 microscope (FEI, Eindhoven, NL) at an accelerating voltage of 120 kV. Images were recorded using a Gatan SIS Megaview IV digital camera (Gatan Inc., Warrendale, PA). Samples were prepared by dropping 20 μL of the suspension of the NPs onto a 300-mesh copper grid and negatively-stained with 20 μL of 1% w/v uranyl acetate.

### 2.2.3 In vitro release study.

To investigate the in vitro protein release profile, PLGA NPs formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS, pH 7.4, to reach the total volume of 2 mL. The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK). Aliquots of 1 mL were collected at specific time points (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5 and 24 h) during the assay, and the withdrawn volume was replaced with pre-heated PBS, pH 7.4, to maintain sink conditions. All the collected aliquots were centrifuged at 14 000 rpm for 15 min, and the

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**Fig. 1** Schematic representation of the microfluidic setup.
spectrophotometric absorbance of the supernatant was read at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

2.2.4 Structural stability of the released proteins. Alterations in the secondary structure of the total protein content released from PLGA NPs were assessed by circular dichroism (CD) spectroscopy. PLGA NPs individually formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS (pH 7.4, 2 mL). The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK) for 24 h. After centrifugation at 14,000 rpm for 15 min, the supernatant was concentrated to 240 μg mL⁻¹ of protein by ultrafiltration using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Bildderica, MA, USA) with a MWCO of 10 kDa. The spectral profile of each sample was compared with the respective native and denatured (90 °C, 1 h) controls. The concentration of native and denatured controls was defined as 1 mg mL⁻¹ and 200 μg mL⁻¹, respectively. The ellipticity value of proteins was scanned over 200–260 nm wavelength using an Aviv Model 400 spectrometer (Aviv Biomedical Inc, Lakewood, New Jersey, USA). A quartz cell was used, and the bandwidth and time-per-point were 1 nm and 1 s, respectively. The mean residue ellipticity (MRE) was calculated according to eqn (2).33

\[
MRE = \frac{\theta_{\text{sample}} - \theta_{\text{media}}}{(N-1) \times 1 \times C} \times MW
\]

where \( \theta \) is the raw ellipticity value (mdeg), MW is the molecular weight of BSA (66.5 kDa), \( N \) is the number of residues of BSA (583), 1 is the cuvette path length (1 mm), and \( C \) is the protein concentration in mg mL⁻¹. The α-helix percentage was also calculated according to eqn (3).34

\[
\alpha\text{-helix} = \frac{-MRE_{400nm} - 4000}{33,000 - 4000} \times 100
\]

2.2.5 Cell culturing. THP-1 cells were grown in suspension in tissue culture flasks (passage 14–25). The cells were maintained in DMEM medium containing FBS (10%, v/v) and penicillin–streptomycin (1%, v/v). Cultures were kept in an incubator (CellCulture CO₂ incubator, ESCO GB Ltd., Downton, UK) at 37 °C with 5% CO₂ in a water saturated atmosphere. After reaching confluency, cells were centrifuged and split into new tissue culture flasks.

2.2.6 Cellular metabolic activity assay. The influence of samples on metabolic activity was assessed in macrophage-like cells differentiated from the THP-1 cell line using the MTT assay. THP-1 cells were seeded in a 96-well plate (1 × 10⁵ cells per well) in 200 μL complete DMEM medium at a PMA concentration of 10 ng mL⁻¹ in order to induce their differentiation into macrophage-like cells.35 After 24 h, cells were washed twice with pre-warmed 1 mL PBS, pH 7.4 and incubated with samples (0.03 μM, determined in relation to the proteins) in 500 μL DMEM for 3 h at 37 °C. After incubation, the cells were washed twice with 1 mL PBS, pH 7.4, and detached with 90 μL trypsin–EDTA. The cells were then washed with 1 mL complete medium and PBS, pH 7.4, through centrifugation at 1300 rpm during 5 min, and fixed with 4% w/v paraformaldehyde (PFA) in PBS, pH 7.4, for 10 min. PFA was removed through centrifugation washes. Finally, cells were resuspended in PBS, pH 7.4, and placed in cytometer tubes. The quantification of proteins associated with macrophage-like cells was done using a FACSAria I cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed using the FlowJo vX.0.7 software.

2.2.7 Cell-nanoparticle interaction. The influence of proteins in their free form or formulated into PLGA NPs on cell association was investigated through flow cytometry analysis in macrophage-like cells differentiated from the THP-1 cell line following a protocol previously developed by Costa et al.36 THP-1 cells were seeded in a 24-well plate (5 × 10⁵ cells per well) in 1 mL complete DMEM medium at a PMA concentration of 10 ng mL⁻¹ in order to induce their differentiation into macrophage-like cells.35 After 24 h, cells were washed twice with pre-warmed 1 mL PBS, pH 7.4, and incubated with samples (0.03 μM, determined in relation to the proteins) in 500 μL DMEM for 3 h at 37 °C. After incubation, the cells were washed twice with 1 mL PBS, pH 7.4, and detatched with 90 μL trypsin–EDTA. The cells were then washed with 1 mL complete medium and PBS, pH 7.4, through centrifugation at 1300 rpm during 5 min, and fixed with 4% w/v paraformaldehyde (PFA) in PBS, pH 7.4, for 10 min. PFA was removed through centrifugation washes. Finally, cells were resuspended in PBS, pH 7.4, and placed in cytometer tubes. The quantification of proteins associated with macrophage-like cells was done using a FACSAria I cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed using the FlowJo vX.0.7 software.

2.2.8 Statistical analysis. The results were represented as mean ± standard deviation from a minimum of three independent experiments. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a post hoc test (Tukey’s honestly significant difference). Differences were considered significant at *p < 0.05, **p < 0.01, or ***p < 0.001. The statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

In this work, PLGA NPs were co-formulated with BSA-FITC and BSA-TRITC via a microfluidic modified-nanoprecipitation technique. The manufacturing of these NPs was performed at 14,000 rpm for 15 min, the supernatant was concentrated to 240 μg mL⁻¹ of protein by ultrafiltration using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Billerdica, MA, USA) with a MWCO of 10 kDa. The spectral profile of each sample was compared with the respective native and denatured (90 °C, 1 h) controls. The concentration of native and denatured controls was defined as 1 mg mL⁻¹ and 200 μg mL⁻¹, respectively. The ellipticity value of proteins was scanned over 200–260 nm wavelength using an Aviv Model 400 spectrometer (Aviv Biomedical Inc, Lakewood, New Jersey, USA). A quartz cell was used, and the bandwidth and time-per-point were 1 nm and 1 s, respectively. The mean residue ellipticity (MRE) was calculated according to eqn (2).33

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\[
\text{Metabolic activity} (\%) = \frac{\text{Experimental value} - \text{NC}}{\text{PC} - \text{NC}} \times 100 (4)
\]
using a X-junction microfluidic chip. An acetone organic phase consisting of PLGA polymer and an aqueous solution of Tween®-80 were used. The standard nanoprecipitation methodology was first described by Fessi et al. who detailed how materials for delivery could be solubilized in the organic phase. In this article, it is shown that the BSA-FITC and BSA-TRITC model proteins can also be included in the aqueous Tween®-80 solution.

The continuous manufacturing of polymeric NPs using microfluidics is a highly versatile technique in which many parameters must be optimized in order to produce the desired particulate system. Therefore, to clarify the manufacturing method, the influence of PLGA concentration in the organic phase and total flow rate of the system was investigated, whilst maintaining the ratio of organic:aqueous solvent phase and aqueous phase concentration of Tween®-80.

An increase in the PLGA concentration of the organic phase from 0.25% w/v to 1% w/v produced a significant increase (*p < 0.05) in the Z-average of NPs from around 110 nm to 140 nm, respectively (Fig. 2A). This increase in size can be explained by the rise in the number and consequent fusion of PLGA chains within the vicinity of nucleation sites during particle formation. PDI values lower than 0.2 across the same range of concentrations (Fig. 2B) demonstrated size distribution uniformity of all NP populations, which is a typical characteristic of microfluidic-produced particles. This is because, the manufacturing device allows the controlled separation of the nucleation and growth stages through the distance away from the location where the initial mixing of the organic and aqueous phase occurs. The ζ-potential, in turn, significantly dropped (**p < 0.01) from around −9 mV to −17 mV with increasing concentrations of PLGA (Fig. 2C) as a result of the higher density of polymer carboxyl endgroups exposed on the surface of the NPs. Increasing concentrations of PLGA also resulted in a considerable increase in the production rate of NPs (Fig. 2D) from around 0.2 g per day to 0.7 g per day. The production rate is usually an undervalued parameter in most bulk and even continuous manufacturing methods used for PLGA NPs production. However, this parameter has attracted a greater focus in recent years. This is because, the production rate directly correlates with the capability of the manufacturing method to address the throughput required for the application in industrial and clinical settings.

These physicochemical properties of NPs are ideal for systemic administration and have been previously shown to permit transit through and escape from the spleen and liver. Furthermore, these properties also permit the application of these particles for cancer nanomedicine. This is because, their ζ-average enables enhanced permeability and retention (EPR) effect by migrating through the vascular fenestrations of tumours. More particularly, the size dimension of the developed NPs could be also useful in the field of brain drug delivery since it is generally accepted that it facilitates the transport of particles through the vascular endothelial cells of the blood–brain barrier. PLGA at 1% w/v in acetone solvent was defined as the organic phase to proceed with further studies, as it provided the highest possible polymer concentration and consequent NPs production rate, whilst maintaining homogeneous monodistributed NPs. The optimization of the manufacturing process involved an increase in the total flow rate from 100 μL min⁻¹ to 2000 μL min⁻¹. These flow rates corresponded to an increase in the organic phase flow rate from 25 μL min⁻¹ to 75 μL min⁻¹ and aqueous flow rate 500 μL min⁻¹ to 1500 μL min⁻¹. Higher total flow rates led to a significant decrease in the Z-average of the PLGA NPs (**p < 0.01, Fig. 3A), which is in accordance with previously published literature by Zhang et al. They have explained that the faster diffusion of the organic phase into the aqueous phase enabled higher nucleation rates and

Fig. 2 Optimization of PLGA concentration for the manufacturing of unloaded NPs, using an organic:aqueous flow rate of 50:150 μL min⁻¹. NP properties of (A) Z-average, (B) PDI and (C) ζ-potential were investigated, as well as the (D) manufacturing productivity. Final conditions were defined as 1% w/v PLGA. Error bars represent the mean ± SD (n = 3) and the significance levels were assigned at *p < 0.05 and **p < 0.01.
the formation of multiple, smaller spatial supersaturation zones. This places the nucleation stage in a privileged position compared to the growth one, since more nuclei are formed and only limited amount of polymer is left to grow them. Therefore, lower diameter NPs can be obtained. The PDI (Fig. 3B) and ζ-potential (Fig. 3C) did not change significantly across the same range of total flow rate, keeping values less than 0.2 and around −20 mV, respectively. Increasing total flow rates resulted in a greater increase in the production rate of the PLGA NPs (Fig. 3D) from around 0.4 g per day to 7 g per day. It is important to highlight that the obtained maximum production rate of 7 g per day demonstrated superior yields to previously reported literature data for polymeric nanosystems, that were manufactured using a single microfluidic device, e.g. 0.0072–0.0144 g per day, 0.012–0.079 g per day, 0.156 g per day and 0.252 g per day. Based on the data presented in Fig. 3, the final total flow rate was defined as an organic : aqueous flow rate of 500 : 1500 μL min⁻¹. These greater flow rates provided the highest productivity, while maintaining homogeneous monodisperse PLGA NPs.

To ensure the controlled, reproducible continuous manufacturing of PLGA NPs using microfluidics, the scale of the manufacturing method was investigated. Specifically, a scale-up of the final yield by ×5 (50 mg) and ×25 (250 mg) was conducted. The ζ-average, PDI and ζ-potential properties did not change significantly and demonstrated to be independent on the scale-up (Table 1), which indicated the strength of the herein proposed continuous microfluidic manufacturing process. This successful up-production in a continuous-flow setup offers potential to speed up the translation of the developed nanomedicines from laboratory to industry and clinical scales.

PLGA NPs were manufactured unloaded, individually formulated with BSA-FITC or BSA-TRITC, as well as co-formulated with BSA-FITC and BSA-TRITC. The ζ-average (around 120 nm), PDI (around 0.1) and ζ-potential (around 15 mV) of all protein-loaded formulations did not exhibit significant changes compared to unloaded NPs (Table 2). PLGA nanosystems individually formulated with either BSA-FITC or BSA-TRITC presented an AE of around 80%. Although there is limited information in the literature highlighting the loading of BSA-TRITC into PLGA NPs, the obtained BSA-FITC AE value was far higher than the ones reported by other authors, approximately around 30–45%. Moreover, the co-formulated nanosystem also presented high AE for both BSA-FITC and BSA-TRITC, corresponding to values of around 70% and 80%, respectively. It was hypothesized that the selected concentration of Tween®-80 endowed the protein-containing aqueous phase with an optimal viscosity to promote its dispersion through the organic phase. This provides enhanced dispersion of BSA-FITC and/or BSA-TRITC molecules through the PLGA matrix. It was furthermore envisaged that any residual Tween®-80 coating the surface of the NPs could act as an entrapment layer for BSA-FITC and/or BSA-TRITC molecules residing at the polymer-surfactant interface.

TEM imaging was used as a tool to provide more insight into the size and morphology of the developed NPs. Monodisperse particles with a homogenous surface and spherical morphology were observed (Fig. 4). All formulations presented a size of NPs of around 100 nm, which is in accordance with DLS measurements.

Protein release was evaluated in vitro in buffered saline (pH 7.4), to represent an intravenous environment. PLGA NPs individually loaded with BSA-FITC were characterized by an initial rapid burst effect, with around 80% of the protein...
being recovered in the first 2 h (Fig. 5A). In comparison, PLGA NPs individually loaded with BSA-TRITC, significantly less of the protein was recovered (around 50%) over the same time period and the overall release was more sustained (Fig. 5B). This results suggested that BSA-FITC may have a tendency to be located close or on the surface of the NPs, whereas BSA-TRITC could be dispersed into the polymeric PLGA matrix.56 Since the TRITC fluorochrome is more hydrophobic than FITC,57 it also rendered BSA-TRITC protein more hydrophobic than BSA-FITC. The PLGA polymer is known to present a hydrophobic backbone and, thus, it is prone to retain hydrophobic and hydrophilic cargos into its matrix and close to its surface, respectively, which can explain the previous release results.56

Co-formulated BSA-FITC and BSA-TRITC PLGA NPs exhibited a release profile of BSA-TRITC comparable to the BSA-TRITC individual formulation, with also around 50% of the protein recovered over the first 2 h (Fig. 5C). However, the release profile of BSA-FITC underwent significant alterations compared to the individual formulation, with only around 20% of the protein recovered over 2 h (Fig. 5C). It was hypothesized that this difference was related to the opposite net charge values of FITC and TRITC fluorochromes. FITC is negatively charged under neutral pH and presents a net value of −1.02, whereas TRITC possesses a net positive charge of +0.99.58 BSA conjugated with the positively charged TRITC, which is hypothesized to be distributed through the polymeric matrix of PLGA NPs, may exert electrostatic interactions with BSA conjugated with the negatively charged FITC, which would be expected to be located closer to the particles’ surface. This electrostatic attraction could therefore be responsible for retaining the BSA-FITC for prolonged time periods, sustaining its release, in comparison to the BSA-FITC individual formulation.

Protein structure is important since alterations in the secondary structure of proteins are likely to be related to their denaturation, which ultimately culminates in aggregation and loss of biologic function.34,59 One of the major disadvantages of the loading of protein therapeutics into polymeric NPs is their denaturation after the manufacturing process, mainly due to the deposition of these molecules in the organic–aqueous phase of emulsions and/or the contact of the organic solvents with their nonpolar amino acids.60 Therefore, maintenance of the secondary structure of proteins after the release from PLGA NPs was studied through CD, where controls of each protein type in its native and denatured form were used for comparison purposes.

All CD spectra presented double minima at around 220 nm and 210 nm, which is characteristic of the α-helical structure of BSA61,62 (Fig. 6). The spectra of BSA-FITC and BSA-TRITC released from the individually and co-formulated PLGA NPs were comparable to the native forms of the proteins. They were also distinguishable from the denatured controls, which produced diminished absorbance bands. The α-helix content was calculated in order to confirm the structural similarities between the released samples and native controls. The calculated values were found in the interval from 50–60% as literature data reports for BSA,34,63 with variations in the α-helix content relative to the control of only around 0%, 4% and 3% for individually loaded BSA-FITC NPs (Fig. 6A) and BSA-TRITC PLGA NPs (Fig. 6B), and co-formulated BSA-FITC and BSA-TRITC PLGA NPs (Fig. 6C). The CD results confirmed that the proposed continuous microfluidic manufacturing method for production of

<table>
<thead>
<tr>
<th>NPs</th>
<th>Z-Average (nm)</th>
<th>PDI</th>
<th>ζ-Potential (mV)</th>
<th>AE (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BSA-FITC</td>
<td>BSA-TRITC</td>
<td></td>
</tr>
<tr>
<td>Unloaded</td>
<td>116.9 ± 1.7</td>
<td>0.105 ± 0.006</td>
<td>−17.2 ± 1.1</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>BSA-FITC</td>
<td>117.4 ± 2.1</td>
<td>0.102 ± 0.012</td>
<td>−14.1 ± 2.3</td>
<td>77.7 ± 4.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>BSA-TRITC</td>
<td>121.3 ± 2.6</td>
<td>0.104 ± 0.002</td>
<td>−15.7 ± 2.1</td>
<td>—</td>
<td>79.9 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>BSA-FITC and BSA-TRITC</td>
<td>118.7 ± 4.1</td>
<td>0.106 ± 0.002</td>
<td>−14.6 ± 2.7</td>
<td>66.3 ± 4.5</td>
<td>77.5 ± 5.7</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5  In vitro release profile of individually loaded (A) BSA-FITC and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Error bars represent the mean ± SD (n = 3).

Fig. 6  CD spectral profile of the total released protein from individually formulated (A) BSA-FITC NPs and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Spectral profile of the respective native and thermally denatured total protein is also represented. Results are presented as mean (n = 3).
polymeric PLGA NPs was successfully able to encapsulate model proteins, either individually or as a co-formulation, in the absence of deviations in the secondary structure of the released proteins.

The influence of the developed nanosystems on metabolic activity was assessed through a MTT assay in macrophage-like cells differentiated from the THP-1 cell line (Fig. 7). This cell line was selected only as a model due to its well described, easy and relatively inexpensive culture. Protein concentrations ranging from 0.0003–0.03 μM were investigated for their effects on metabolic activity. The metabolic activity of macrophage-like cells was found to be always above 70% when in contact with the free BSA-FITC, BSA-TRITC, and mixed BSA-FITC and BSA-TRITC at all evaluated concentrations. This demonstrated the absence of cytotoxicity associated with the free proteins and was expected since the major molarity contribution from the protein-fluorochrome complex originates from the BSA, for which the normal physiologic concentration is much higher than the maximum one herein tested (around 0.76 M in blood serum). Unloaded NPs also exhibited cell metabolic activity greater than 70% for all tested concentrations, as well as NPs individually loaded with BSA-FITC or BSA-TRITC and co-formulated with BSA-FITC and BSA-TRITC. The polymeric matrix of NPs did not induce cytotoxicity and did not compromise cell viability. This observation was also true for the physical mixture of NPs individually loaded with BSA-FITC or BSA-TRITC in amounts equivalent to the co-formulated NPs. Therefore, it is important to note, according to the 10993:5 norm from the International Organization for Standardization (ISO) for in vitro pre-clinical evaluation of the cytotoxicity of medical devices, these PLGA NP formulations may be considered non-toxic and potentially safe.

It is well reported that the hydrophilicity of proteins is one of the main characteristics responsible for their poor interaction with the hydrophobic lipid bilayer of cells. This may be especially critical for protein-based drugs that require an efficient intracellular transport and delivery to exert a certain therapeutic effect. Therefore, the potential of the developed NPs to improve cell–protein interaction was evaluated in macrophage-like cells differentiated from the THP-1 cell line (Fig. 8).

The loading of BSA-FITC into PLGA NPs did not appear to improve cell interaction, with no significant differences observed in comparison to the free BSA-FITC (Fig. 8A). This correlated to the release studies (Fig. 5A) that suggested that BSA-FITC may be preferably located on the surface of the NPs which, as a result, may adopt hydrophilic properties that may hinder cell association. Whereas, the loading of BSA-TRITC into PLGA NPs significantly improved (**p < 0.001) cell interaction compared to the free BSA-TRITC (Fig. 8B). This again confirmed observations from the release studies (Fig. 5B) and suggested that the more hydrophobic BSA-TRITC protein may be preferably dispersed through the polymeric core of PLGA NPs, rather than on their surface. The hydrophilic properties of BSA-TRITC were therefore camouflaged by the hydrophobic ones of the PLGA NPs, hence favouring cell interaction. These findings were in accordance with research published by Miklavžin et al., who showed that the complexion of erythropoietin with polyelectrolytes to render a more hydrophobic complex was able to enhance the interaction and consequent permeability of the protein through Caco-2 cell monolayers. Apart from the hydrophilicity, it was hypothesized that the developed PLGA NPs might profit from residual Tween®-80 molecules on their surface to bind the scavenger receptor of macrophage-like cells, hence promoting an enhanced cell–protein interaction. Tween®-80 has the capacity to adsorb apolipoprotein E, which is a known ligand of this type of cell receptor and thus mediates cell-NP binding. Co-formulated PLGA NPs significantly increased the cell association of co-formulated BSA-FITC and BSA-TRITC (**p < 0.01), compared to the mix of the free form of the proteins (Fig. 8C). This finding highlights an important advance in this proof-of-concept study due to the usefulness of developing a delivery system capable of enhancing protein co-delivery. The co-formulated PLGA NPs demonstrated a significantly higher cell association (**p < 0.001) compared to the physical mixture of NPs individually loaded with BSA-FITC and BSA-TRITC (Fig. 8C), which reinforced the superior biologic performance of the all-in-one nanosystem.
4. Conclusion

A continuous microfluidic manufacturing method that enabled the engineering of monodisperse BSA-FITC and BSA-TRITC co-formulated PLGA NPs with a high association efficiency is reported herein. The enhanced production rate and scale-up potential of the demonstrated microfluidic method indicated the capacity to manufacture monodisperse PLGA NPs in industrial and clinical batch sizes. The microfluidic manufacture process preserved the protein secondary structure and did not appear to induce denaturation upon release. Co-formulated BSA-FITC and BSA-TRITC PLGA NPs did not present cytotoxicity and significantly enhanced the association of BSA-FITC and BSA-TRITC with macrophage-like cells compared to the free mixed form of the proteins and even the physical mixture of NPs individually loaded with each protein type. Therefore, it is anticipated that this model study will pave the way towards novel combination therapies based on PLGA nanosystems that require the co-delivery of protein therapeutics to a range of pathologies. Perspectives for future work include the co-formulation of other proteins and investigation of the preservation of bioactivity post-release, as well as studies of the efficacy of the nanosystem in vivo.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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