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# Design of extracellular protein based particles for intra and extra-cellular targeting

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ABSTRACT

In recent years, a significant amount of effort has been dedicated to the development of both nano- and micro-technologies as they offer endless possibilities with regards to their biological applications. At the forefront of these technologies is the synthesis of particles for various applications in targeted drug and growth factor delivery, gene therapy, medical imaging and tissue engineering. In this study we used the layer-by-layer self-assembly method to synthesis protein-based microparticles using extracellular matrix proteins, such as collagen and fibronectin, in a simple and scalable way. Particle characterisation was performed using fluorescence microscopy, zeta potential analysis and scanning electron microscopy. Furthermore, two different cell types were used to investigate microparticle toxicity, attachment and/or internalisation. The results obtained not only showed a significant reduction in the cytotoxicity of these protein-based particles but also a significant increase in their attachment and internalisation by cells compared to their polymeric counterparts. In addition, we provide evidence for use of such particles in achieving the sustained release of Bone Morphogenic Protein-2 which is widely used for bone tissue engineering. This study has implications in the development of functional, biocompatible and non-toxic particles for intra and extra-cellular targeting and sustained release of various drugs, growth factors and genetic materials for numerous applications in medicine.



## 1. Introduction

In recent years, a significant amount of effort has been dedicated to the development of both nano- and micro-technologies as they offer endless possibilities with regards to their biological applications. At the forefront of these technologies is the synthesis of particles for various applications in targeted drug delivery [1,2], growth factor delivery [3,4], gene therapy [5], medical imaging [6] and tissue engineering [7,8]. Delivery of drugs via nano or microparticles aims to reduce collateral damage to healthy tissues, extend the drug's availability and effectiveness at the site, and then finally it is hoped that the particles will degrade naturally after carrying out their function [9–11]. Drugs that are contraindicated for administration as a free drug can be loaded within these particles so as to reduce any potential systemic side effects [10,12].

This has led to various synthetic and biological materials to be investigated for possible uses in nano and micro particle synthesis. Examples of such materials include, but are not limited to, gold [13], zirconia [14], iron oxide [15], $TiO_2$ and polystyrene [16]. The problem with using such materials is that they suffer in terms of biodegradability and biocompatibility. Newer carbon-based materials such as graphene and carbon nanotubes had shown promise in their applications in biology due to their unique properties and size [17,18]. These materials however had two fundamental flaws; their susceptibility to immune responses due to their composition and the growing concerns about their potential toxicity as they may be retained within the body [17,19]. To overcome this problem, these materials can be coated with proteins and polysaccharides, which naturally exist in the extracellular compartment of the body.

One of the methods of synthesizing nano or micro particles for biological applications is the layer-by-layer (LbL) self-assembly process. In this study, it is used primarily due to its simplicity and ease of being carried out [20–23]. The "layers" used in LbL synthesis often consist of oppositely charged polyelectrolyte solutions in order to create a coating that is held together by electrostatic forces of attraction [21,24]. LbL synthesis can be further applied to take advantage of other interactive forces between a range of materials such as electrostatic interactions, hydrogen or covalent bonding and hydrophobic interactions among others [25,26]. To a certain extent, any combination of materials can be used, be it biological or synthetic or a hybrid of the two, as long as they are able to interact by any of the aforementioned interactive forces [21,23,27,28].

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Another advantage of LbL synthesis is the fact that it allows for the size of the particles synthesized to be easily modified by using sacrificial templates of different sizes and shapes or by adding different number of layers. Usually, the most commonly used sacrificial templates onto which particles are synthesized are silica or polystyrene solid spheres, due to the ease of removal to achieve hollow particles [21,29]. A range of spherical and tubular nano and micro particles have been synthesized using templates of varying sizes and materials such as polystyrene sulfonate (PSS), polyallymine hydrochloride (PAH) and polyacrylic acids (PAA) [22,23,30–33]. However, use of such particles in medicine is limited due to their reduced biocompatibility and biodegradability. For this reason increasing interest has grown in synthesizing particles using materials that are native to the body, such as protein-based polyelectrolyte which have a similar composition to the extracellular matrix (ECM) [34]. Use of such materials aims to improve cell-particle interactions and promote cell adhesion and proliferation without compromising their biocompatibility [35–37].

Previously, proteoglycans, glycosaminoglycans and proteins such as collagen (COL) [38], hyaluronic acid [39], chitosan [33] and alginate [40] were used to create more biologically functional multilayer's. We have also previously shown successful synthesis of tubular nano and micro size structures using ECM proteins such as collagen [41]. These proteins however were not used to synthesize spherical microparticles before. ECM proteins such as Collagen (COL) and Fibronectin (FN) are an essential component of ECM and play an important role in cellular functions, aiding cell growth, proliferation and differentiation. Structurally, both COL and FN can interact and bind to one another via specific binding sites and promote cell adhesion and attachment by engaging with specific cell integrins [42–45].

In this study, we report synthesis of COL and FN based microparticles using template-based LBL self-assembly process. We have characterized these particles using various microscopy techniques such as fluorescence, light and scanning electron microscopy. Changes in particle charges as a result of protein coating are quantified using zeta potential analysis. *In vitro* toxicological studies were carried out using two different cell lines; human dermal fibroblast (HDF) and colorectal adenocarcinoma (HT29) cells. Cellular interactions with these particles, their internalization and effect on metabolic activity were evaluated using fluorescence microscopy and Alamar blue assay. To the best of our knowledge, this is the first example of synthesis and application of self-assembled protein-based spherical microparticles and their *in vitro* evaluation. A proof of concept study utilizing these particles to achieve the sustained release of growth factors such as Bone Morphogenetic Protein-2 (BMP-2) is also presented.

The method to produce protein-based particles described in this study is easy, scalable and provides novel opportunities in design and application of biocompatible particles for targeted drug and growth factor delivery via scaffolds for applications in biomaterials and tissue engineering.

## 2. Materials and methods

### 2.1. Materials and reagents

Poly(sodium-*p*-styrenesulfonate) (PSS) and poly(fluorescein isothiocyanate allylamine hydrochloride) (Flu-PAH), acetic acid, Tris buffered saline, paraformaldehyde, Alamar blue, Trypsin, Sodium Acetate, and T-75 (75 $cm^2$) flasks were all purchased from Sigma–Aldrich (UK). Collagen type 1 (COL, rat-tail) was purchased from Roche Diagnostics GmbH (Germany) and Human plasma fibronectin (FN, 1 mg/ml) was purchased Millipore Temecula, CA (USA). Red-fluorescent COOH-coated polystyrene and poly(lactic acid) particles (Red-F and PLA-redF respectively) with a diameter of 3 μm and 2 μm respectively were purchased from Micromod Partikeltechnologie GmbH (Germany). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12 low (1 mg/ml) and high glucose (4.5 mg/ml)), Dulbecco's Modified Eagle Medium (DMEM low (1 mg/ml)), GlutaMAX, Phosphate Buffered Saline (PBS), Foetal bovine serum (FBS) and penicillin-streptomycin (PenStrep) solution, were all purchased from Gibco. Rhodamine Phalloidin and DAPI were purchased from Life Technologies. Recombinant BMP-2 and BMP-2 Quantikine ELISA Kit were purchased from R&D Systems. Human Dermal Fibroblast (HDF) and HT29 colorectal adenocarcinoma (HT29) cell lines were obtained from The European Collection of Cell Cultures (ECACC). Bone Marrow Derived human Mesenchymal Stem Cell (hMSC) cell line was obtained from Lonza.

## 3. Methods

### 3.1. Preparation of polyelectrolyte solutions

The protein-based solutions used for the LbL approach were 0.2 mg/ml COL and FN solutions. The COL and BMP-2 (2 μg/ml) solution was prepared using 100 mM acetate buffer and the FN solution was prepared with 50 mM Tris buffer. The polymer-based solutions used were 1 mg/ml PAH and PSS polyelectrolyte solutions. Both solutions were prepared using 100 mM acetate buffer.

### 3.2. Preparation of protein and polymer based microparticles

Microparticle synthesis involved the use of carboxyl-functionalized polystyrene particles as a template core (Φ 3 μm). These cores were alternatively coated with two different layers of polyelectrolytes consisting of either polymers or proteins, using the LbL approach. In our experiment, we used fluorescently-labeled PAH (Flu-PAH) and PSS as our polymeric and COL, FN and BMP2 as our protein layers. The process used involves submersion of the particles in a cationic solution (COL, PAH or BMP2), briefly sonicating them to ensure even coating and then placing them on agitation on a tube roller at room temperature (RT) for 10 min. Particles were collected by centrifuging them at 10,000 revolutions per minute (rpm), at RT for 5 min to form a pellet, which was later washed with a buffer solution to remove excess polyelectrolyte solution before submerging them in an anionic solution (FN or PSS). These steps were repeated as necessary to achieve coated microparticles with the desired number of bilayers (Fig. S1). On completion of microparticle synthesis, all the particles were suspended in deionised water after the final washing step. The exact microparticle composition of each microparticle synthesised for this study is shown seen in Table 1.

### 3.3. Preparation of fluorescently-labeled protein microparticles

FITC-labeled polyelectrolyte was used during LBL assembly over Rhodamine labeled core particles so that when LBL assembly is successful, the particle turns to yellow and can be visualized by a fluorescent microscope, providing immediate confirmation of the self-assembly process as illustrated in Fig. 1. The composition of the microparticles synthesized and their abbreviations can be found in Table 1.

### 3.4. Characterization of particles using fluorescence microscope

All imaging was carried out using Leica DMI4000b fluorescence microscope and the images processed using LAS AF software. For microparticle characterization, a drop of the particle solution was placed on a glass slide and the samples were left to dry before being