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Magnetic-based multi-layer microparticles for endothelial progenitor cell isolation, enrichment, and detachment

Aniket S. Wadajkar [a,b], Sonia Santimano [a,b], Liping Tang [a,b], Kytai T. Nguyen [a,b,*]

[a] Department of Bioengineering, The University of Texas at Arlington, Arlington, TX 76019, USA
[b] Joint Biomedical Engineering Program between The University of Texas at Arlington and The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

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ABSTRACT

Although endothelial progenitor cells (EPCs) are useful in many applications including cell-based therapies, their use is still limited due to issues associated with cell culture techniques like a low isolation efficiency, use of harmful proteolytic enzymes in cell cultures, and difficulty in *ex vivo* expansion. Here, we report a tool to simultaneously isolate, enrich, and detach EPCs without the use of harmful chemicals. In particular, we developed magnetic-based multi-layer microparticles (MLMPs) that (1) magnetically isolate EPCs via anti-CD34 antibodies to avoid the use of Ficoll and harsh shear forces; (2) provide a 3D surface for cell attachment and growth; (3) produce sequential releases of growth factors (GFs) to enrich *ex vivo* expansion of cells; and (4) detach cells without using trypsin. MLMPs were successful in isolating EPCs from a cell suspension and provided a sequential release of GFs for EPC proliferation and differentiation. The cell enrichment profiles indicated steady cell growth on MLMPs in comparison to commercial Cytodex3 microbeads. Further, the cells were detached from MLMPs by lowering the temperature below 32 °C. Results indicate that the MLMPs have potential to be an effective tool towards efficient cell isolation, fast expansion, and non-chemical detachment.

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1. Introduction

Stem cells such as endothelial progenitor cells (EPCs) have great promise as a renewable source of cells for basic and applied research. For instance, cell-based therapies use EPCs to treat patients with cardiovascular diseases including ischemic heart disease, in-stent restenosis, and peripheral arterial occlusive disease [1,2]. EPCs differentiate into endothelial cells (ECs) and contribute to tissue repair and neovasculature homeostasis by participating in angiogenesis and arteriogenesis [3]. EPCs can be isolated from one of their major sources including peripheral blood, umbilical cord blood, embryo, and bone marrow. However, a major limitation with the EPCs is their limited availability in the sources. For example, the number of EPCs present in peripheral blood of healthy humans is only 0.01%–0.0001% of total mononuclear cells [4]. Moreover, it requires several days of *in vitro* cultures to produce a great enough number of EPCs to be used in cell-based therapies [4,5].

Several cell isolation and expansion techniques have been developed to generate enough numbers of cells including stem cells for cell-based therapies. Cell isolation methods such as Ficoll-Paque gradient centrifuge [6], fluorescence-activated cell sorting (FACS) [7], magnetic-activated cell sorting (MACS) beads [8] have been used extensively over the last decade. In addition to cell isolation, various cell expansion technologies including microbeads like Cytodex3 microbeads [9] for cell expansion have been developed. These techniques have shown some degree of success, but can be used only for a single purpose, either cell isolation or cell expansion. In addition, each of these procedures is hampered by serious limitations. In particular, harsh chemicals, high shear forces, low isolation efficiency, and elaborate culture time is often associated with the Ficoll-Paque gradient centrifuge for cell isolation [6]. FACS requires fluorescent labeling of the cells and the equipment is very expensive [7]. Further, MACS beads do not support cell expansion and do not provide any proliferation or differentiation growth factors (GFs) [8]. Finally, Cytodex3 microbeads cannot be used for cell isolation, do not provide proliferation or differentiation GFs, and require harmful proteolytic enzymes for cell detachment [9]. In general, all the cell expansion techniques use trypsin and ethylenediamine tetraacetic acid

* Corresponding author. 500 UTA Blvd, ERB 241, Arlington, TX 76019, USA. Tel.: +1 817 272 2540; fax: +1 817 272 2251.
E-mail addresses: aniket.wadajkar@mavs.uta.edu, knguyen@uta.edu (K.T. Nguyen).

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(EDTA) that affect the cellular functionality through every passage by cleaving the cellular proteins [10]. In an effort to avoid the use of proteolytic enzymes, Tamura et al. [11] developed poly(*N*-isopropylacrylamide)-grafted polystyrene microbeads to support cell adhesion and detachment in response to temperature alteration. However, these microbeads are not useful for cell-selective isolation from a crude sample and do not provide enrichment GFs for cell expansion.

Therefore, to overcome the limitations of currently available cell isolation and expansion techniques and owing to the advantages of EPCs, we report simultaneous cell separation, proliferation, differentiation, and detachment on a 3D surface of magnetic-based multi-layer microparticles (MLMPs). The aim of research was to develop MLMPs that can be used for magnetic EPC separation, enrichment, and detachment. The MLMPs contain four layers as shown in Fig. 1: (1) CD34 antibodies at the particle surface for selectively targeting EPCs in a cell mixture or blood/crude sample. The identified unique cell surface markers for EPC isolation include CD34, CD133/AC133, vascular endothelial growth factor (VEGF) receptor 2, and kinase domain receptor. Of these, CD34 is the most frequently used marker [12,13]. (2) An outer shell of thermo-responsive poly(*N*-isopropylacrylamide-co-allylamine) (PNIPA-AH) to provide a surface for cell attachment and detachment depending on temperature alteration. At temperatures higher than its lower critical solution temperature (LCST, 32–34 °C), PNIPA-AH becomes hydrophobic, supporting the cell adhesion, whereas at temperatures below the LCST, PNIPA-AH becomes hydrophilic, detaching the cell non-invasively without using proteolytic enzymes. This layer also contains VEGFs which will rapidly be released for EPC proliferation [14,15]. (3) Iron oxide magnetic nanoparticles (MNPs) add magnetic content to the MLMPs for magnetic cell separation, and (4) a biodegradable core of poly(lactide-co-glycolic acid) (PLGA) microparticles containing basic fibroblast GFs (bFGFs) will eventually be released as PLGA degrades for EPC differentiation [15,16]. The hypothesis is that, when mixed with a cell suspension, only EPCs attach to the MLMPs surface; the cell-particle complexes can be isolated using an external magnetic force; the isolated cells can adhere to the outer layer of PNIPA-AH when incubated at 37 °C; a rapid release of VEGFs from PNIPA-AH and a slow and sustained release of bFGFs from PLGA can enrich EPCs to generate a large-scale suspension culture; and the cells can be detached from the PNIPA-AH surface at the temperatures below the LCST.

2. Experimental section

2.1. Synthesis of MLMPs

All the chemicals were purchased from Sigma–Aldrich and used without further purification, if not mentioned. GFs-loaded MLMPs were synthesized by a layer-by-layer approach. First, PLGA microparticles were synthesized by a double emulsion technique [17,18]. Briefly, aqueous solution of bFGFs (8 ng/ml, 1 ml) was added drop-wise to the oil phase of PLGA (2% w/v, 50:50, with carboxyl end groups, Lakeshore Biomaterials, Birmingham, AL) solution in dichloromethane (5 ml). The resultant primary emulsion solution was then added drop-wise to the aqueous solution of polyvinyl alcohol (0.5% w/v, 20 ml) to form microparticles. After allowing solvent evaporation, the particles were washed via centrifugation (1000 rpm for 5 min for each wash, thrice), collected via freeze-drying, and stored at −20 °C for further use. The wash samples (supernatants) were collected to quantify bFGFs loading efficiency.

Second, iron oxide MNPs (Meliorum technologies, Rochester, NY) were functionalized with amine and silane groups as previously described [19,20]. Briefly, MNPs (10 nm diameter, 74.24 mg) were dispersed in a mixture of DI water and ethanol (1:99) by sonication at 30 W. After 10 min, acetic acid (3 ml) was added, sonication was continued for 10 min, reaction was transferred to magnetic stir plate, to which (3-aminopropyl)trimethoxysilane (283.1 μl) and vinyltrimethoxysilane (243.5 μl) were added, and the reaction was stirred vigorously for 24 h. The particles were washed thrice with a mixture of DI water and ethanol. Surface functionalized MNPs were then conjugated to the PLGA microparticles by carbodiimide chemistry [21]. Briefly, PLGA microparticles (20 mg) were added to 2-(*N*-morpholino)ethanesulfonic acid buffer (MES, 0.1 M, 90 ml), stirred for 20 min, and then *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 160 mg) and *N*-hydroxysuccinimide (NHS, 160 mg) were added. Meanwhile, in a separate beaker, surface functionalized MNPs (14 mg) were sonicated with MES buffer (5 ml) at 40 W and then added drop-wise to the reaction, followed by the addition of sodium dodecyl sulfate (SDS, 2 mg), and the reaction was stirred for 6 h. MNPs-conjugated PLGA microparticles were washed thrice with DI water, collected using a magnet, freeze-dried, and stored at −20 °C for further use.

Finally, *N*-isopropylacrylamide (NIPAAm) and allylamine (AH) were copolymerized on the surface of MNPs-conjugated PLGA microparticles by radical polymerization [21,22]. Briefly, MNPs-conjugated PLGA microparticles (28 mg) were sonicated with DI water (90 ml) at 40 W for 10 min, transferred to a magnetic stir plate, and NIPAAm (300 mg), AH (300 μl), *N*,*N*′-methylenebisacrylamide (BIS, 10.5 mg), and SDS (1.5 mg) were added to the reaction. After 10 min, ammonium persulfate (APS, 30 mg) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 39 μl) were added and the reaction was stirred for 4 h in N_2 environment. MLMPs were washed thrice with DI water, collected using a magnet, and freeze-dried. Further, VEGFs were loaded in the PNIPAAm-AH layer by adding aqueous solution of VEGFs (8 ng/ml, 0.5 ml) to MLMPs (20 mg) in DI water (5.5 ml). The solution was placed on a shaker at 4 °C for 3 days to allow VEGFs absorption in the PNIPAAm-AH. After 3 days, VEGFs-loaded MLMPs were collected by a magnet and the supernatant was collected to quantify VEGFs loading efficiency indirectly.

2.2. Particle characterization

The particles were characterized at each step of the synthesis procedure by particle sizer with dynamic light scattering (DLS, Brookhaven Instruments, Holtsville, NY) detector and scanning electron microscope (SEM, S-3000N, Hitachi,

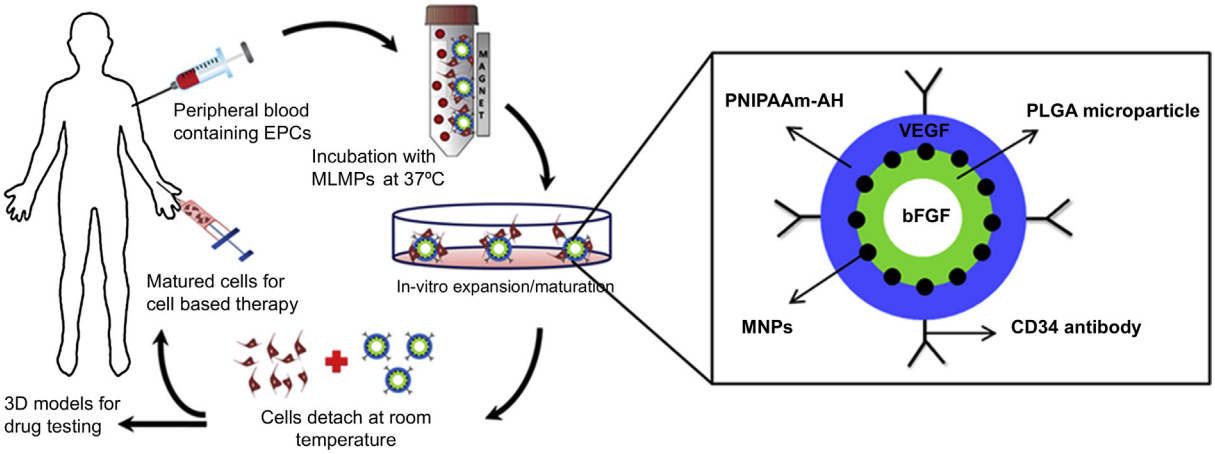

Fig. 1. Schematic of MLMP application in EPC isolation, enrichment, and detachment (left). Magnified schematic of MLMP depicting layer-by-layer assembly of antibodies, polymers, GFs and MNPs (right).

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