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Human endothelial progenitor cell attachment to polysaccharide-based hydrogels: A pre-requisite for vascular tissue engineering

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Abstract A hydrogel was prepared from polysaccharides (pullulan/dextran/fucoidan) and evaluated as a novel biomaterial for Endothelial Progenitor Cell (EPC) culture. Using a cross-linking process with sodium trimetaphosphate in aqueous solution, homogeneous, transparent and easy to handle gels were obtained with a water content higher than 90%. Circular scaffolds (6 mm diameter and 2 mm thickness discs) were used for cell culture. Different types of EPCs were used: CD34+ derived ECs from cord blood and two sorts of CD133+ derived ECs from human bone marrow, old (30 days) and young (4 days) cells. EPCs were characterised as endothelial cells by immunofluorescent stainings for CD31 and Dil-Ac-LDL. CD133+ derived ECs from bone marrow were characterized by RT-PCR for CD31, VE-cadherin and KDR. HSVECs (Human Saphenous Vein Endothelial Cells) were used as control cells. We evaluated whether different kinds of EPCs could adhere on this novel hydrogel 4 h and 24 h after seeding, by a colorimetric quantitative test. EPCs adhered to hydrogels in serum- free conditions with values being over than 80% for young CD133+ cells at 4 h and 24 h. This pullulan-based hydrogel could constitute a suitable support for vascular cell adhesion as a pre-requisite for vascular tissue engineering.

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1 Introduction

Despite numerous attempts all over the world, through different approaches over more than one decade, the engineering of a small-caliber blood vessel substitute for use in peripheral and coronary bypass surgery is still a challenge. As for a normal blood vessel, non-thrombogenicity is provided by the vascular endothelium, thus, to construct blood vessels by tissue engineering approach one technique used is the seeding of Endothelial Cells. At present, EC seeding of synthetic prostheses is performed in humans with autologous EC [1], requiring the harvesting of cells, followed by culturing and amplification for several weeks and the seeding onto coated grafts. Because large amounts of seed cells, such as ECs, are needed, to completely cover the inner surface of graft, stem cell technology could be useful in vascular tissue engineering [2], because one therapeutic clinical perspective would be to transplant endothelial progenitor cells for in vitro endothelialisation of grafts. Indeed, the isolation of endothelial progenitor cells from human peripheral and umbilical cord blood [3] and from human bone marrow [4, 5] generated a great hope in the fields of cellular therapies and regenerative medicine.

On the other hand, besides the endothelialisation procedure and the type of cell, the search for biocompatible materials and scaffolds that could be seeded with cells for vascular engineering was a matter of concern. With this purpose, biodegradable polysaccharide-based hydrogels were developed [6, 7]. Bovine collagen-based scaffolds provided a physical/structural support and have been used for *in-vitro* cell culture. Synthetic polyethylene glycol (PEG)-based hydrogels have been also studied as scaffolds for vascular cell culture. However, these materials have been shown to be intrinsically resistant to protein adsorption

and chemical modification or surface coating with adhesion molecules were required for cell adhesion [8].

In this study, we used a mixture of three polysaccharides as a material to develop a novel polysaccharide-based hydrogel. Pullulan is a neutral, linear and non-immunogenic polysaccharide, produced from starch fermentation by the fungus *A. pullulans* [9]. It consists of glucose units linked through α 1,6- and α 1,4-glucosidic bonds and has been widely used in the food, pharmaceutical and cosmetic industries. Moreover, pullulan is attractive as a biomaterial because of its good mechanical properties and biocompatibility [10].

A semi-synthetic dextran with a high molecular weight was also selected for the preparation of the hydrogel. Fucoidan, a sulfated polysaccharide extracted from brown algae, was previously reported to improve endothelial cell proliferation and tube formation *in vitro* in association with VEGF (Vascular Endothelial Growth Factor) [11]. Moreover, low molecular weight fucoidan enhances neovascularization in a rat ischemia model [12].

The present study was designed to evaluate the suitability of this novel hydrogel prepared by a cross-linking of polysaccharides to support the attachment of human ECs of different origins, as a precursor to further studies involving cell proliferation and differentiation, as well as exposure to shear stress.

2 Materials and methods

2.1 Isolation and culture of endothelial cells and their progenitors

Human umbilical cord blood, human bone marrow and human saphenous veins were collected from donors according to the French Legislation.

Human umbilical cord blood samples were immediately processed in the laboratory for isolation of EPCs according to the procedure described by Bompais et al. [13] with some modifications. In short, mononuclear cells were separated on Lymphocytes Separation Medium (density 1.077; Eurobio, Les Ullis, France) and the collected cells extensively washed before being seeded in a culture flask in RPMI 1640 (Life Technologies, Cergy Pontoise, France) supplemented with 10% Fetal Calf Serum (FCS) (Sigma, Saint Quentin Fallavier, France) for 2–4 h. Then, the non-adherent fraction was collected and centrifuged and the CD34+ cells were isolated using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Paris, France). The selected CD34+ cells were seeded on gelatin-coated flasks in an EGM-2-MV BulletKit medium (Cambrex, Emerainville, France). Clusters of endothelial cells appeared after approximately 7–10 days and the cells were classically amplified up to passage 6 to 10.

Concerning human bone marrow, mononuclear cells were separated on Histopaque®, washed, centrifuged, then CD133+ were isolated with immunomagnetic beads (Miltenyi Biotec, France), plated onto gelatin coated wells and cultured in specialised media according to the procedure described by Bagley et al. [5] with some modifications. In short, CD133+ cells were cultured in medium supplemented with 50 ng/mL VEGF (R&D Systems) and 10 ng/mL rhbFGF (PromoKine®).

Finally, mature endothelial cells were isolated from human saphenous veins (HSVECs) collected after surgical coronary bypasses. The samples were kept in Hank's Balanced Salt Solution supplemented with heparin (50 UI/mL) and transferred to the laboratory for isolation of ECs. The procedure is that described by Fernandez et al. [14]. The isolated cells were seeded in culture flasks coated with gelatin in complete medium. Cells from passages 3 to 6 were used in experiments. HSVECs were used as control cells.

2.2 Endothelial cell characterisation

EPCs were characterised as endothelial cells by immunofluorescent stainings for CD31 and Dil-Ac-LDL. Cells seeded on gelatin-coated 4-wells culture plates were fixed by 1% paraformaldehyde (PFA), blocked for 1h in PBS − 2% BSA − 0.01% glycine and sequentially incubated with primary antibodies against CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with the appropriate secondary antibodies labelled with Alexa 488 (10 μg/mL; Molecular Probes, Cergy Pontoise, France). To detect acetylated low-density lipoprotein (Dil-Ac-LDL) uptake, cells were incubated with 2 μg/mL of Dil-Ac-LDL (Arbor Bio-Products, Norwood, MA, USA) for 24 h before being fixed with 1% paraformaldehyde. Images were taken using the 40 X objective on a fluorescent Zeiss Axiovert 25 microscope (Seli, Toulouse, France) equipped with an Olympus DP70 camera (Seli, Toulouse, France) and using the AnalySIS® software program provided by Soft Imaging System® (Münster, Germany).

Moreover, CD133+ derived ECs from bone marrow were characterised by Reverse Transcriptase PCR (RT-PCR) for KDR (kinase insert domain receptor, termed also Vascular Endothelial Growth Factor Receptor-2 (VEGFR2) or Flk1); CD31 (PECAM) and VE-cadherin. First strand cDNA was synthesized by RT of 2.5 μg total RNA isolated from HSVECs or CD133+ derived ECs using RNeasy total RNA kit (Qiagen) and amplified by Taq DNA polymerase dissolved in PCR buffer (GIBCO, BRL) in a 25 μL reaction containing 10 μM of primers (Table 1) (GENSET Oligos). The PCR profile consisted of 2 min of denaturing at 95°C, followed by 35 cycles of 45 s of denaturing at 94°C, 40 s of annealing at 61°C, 1 min of extension at 72°C, and a final extension step of 3 min. The PCR product (10 μL) was

Table 1 List of the different primer pair sequences that were used for RT-PCR with the forward and reverse sequences, the optimal temperature and the length of the PCR products; CD31, VE-cadherin (Vascular Endothelial-cadherin), KDR (VEGFR-2), Po (housekeeping gene)

Oligonucleotide	Sequence	Tm	Length of PCR procduct (bp)
CD31	upper: CAGCCTTCAACAGAGCCAACC	56.2°C 52.1°C	116
	lower: CACTCCGATGATAACCACTGC		
Po	upper: ATGCCCAGGGAAGACAGGGC	59.8°C 55.3°C	166
	lower: CCATCAGCACCACAGCCTTC		
KDR	upper: AAGTGGAGGCATTTTTCATAA	48.6°C 49.5°C	228
	lower: CATAAGGCAGTCGTTCACAAT		
VE-Cadherin	upper: GGCTCAGACATCCACATAACC	51.0°C 57.3°C	145
	lower: CTTACCAGGGCGTTCAGGGAC		

separated by a 1% agarose gel and stained with ethidium bromide to identify products. We have chosen Po (Homo sapiens, ribosomal protein, locus BC015690) as the reference gene which encodes for a ribosomal protein, and which is not influenced by the experimental conditions. Table 1 shows the different primers used with the temperature and the length of PCR product.

2.3 Scaffold preparation

Polysaccharide-based hydrogels were prepared using a mixture of pullulan/dextran/fucoidan 71:24:5 with a total concentration of 25% (w/v) (Pullulan, MW 200,000, Hayashibara Inc.; Okayama, Japan; Dextran MW 500,000, Pharmacia, Kalamazoo, USA; Fucoidan, Sigma, St. Louis, USA). Chemical cross-linking of polysaccharide was carried out using the cross-linking agent sodium trimetaphosphate STMP (11% w/w, Sigma, St Louis, USA) under alkaline conditions. Polysaccharide solution mixed with STMP was poured into Petri dishes then incubated at 50°C for 15 min and 2 mm thick gel pieces were obtained. A circular punch (diameter 6 mm) was then used to obtain small discs suitable for cell culture. Resulting gels were washed extensively with phosphate buffer saline (PBS pH 7.4), sterilized under UV light then stored in PBS at 4°C until use. In some cases, scaffolds were coated with gelatin (1% w/v).

2.4 *In vitro* cell attachment test

CD 34+ derived ECs, CD133+ derived ECs (30 days old or freshly isolated ones 4 days old) and HSVECs were seeded on hydrogels plated in 96-wells plates (seeding density: $90 \times 10^3/cm^2$), in medium DMEM without phenol red and incubated for 4 and 24 h at 37°C (4 wells per cell series and surface). For CD34+ derived ECs and HSVECs pools were used for cell seeding while for CD133+ derived ECs, two different donors were concerned separately and were not pooled. At the end of the incubation period, the quantitative colorimetric attachment tests were performed to assess cell attachment as previously described by Verrier et al. [15]. Briefly, endothelial cells attachment could be quantified by absorbance measurement with a direct relationship between the number of cells and N-acetyl-β-D-hexosaminidase enzymatic activity. Results were compared with controls, ie culture plates coated with gelatin (1% (w/v), (Sigma, St. Louis, USA) without hydrogel.

2.5 Statistical analysis

The U-Mann-Whitney non parametric test was used for statistical analysis. $p < 0.05$ was considered as significant.

3 Results

Phase contrast micrographs of EC cultures are presented in Fig. 1(A)–(C) showing the typical cobblestone morphology of ECs. Concerning the 30 days cells, a previous monolayer of spindle-shaped cells was observed at day 15 (data not shown). Figure 1(D) shows 4 days old CD133+ selected from bone marrow cells.

To confirm the EC phenotype, the cells were analysed by immunostaining as well as by the mRNA expression.When compared to mature human saphenous vein endothelial cells (HSVECs), CD34+ derived ECs and CD133+ derived ECs in the same culture conditions are positive for CD31 and

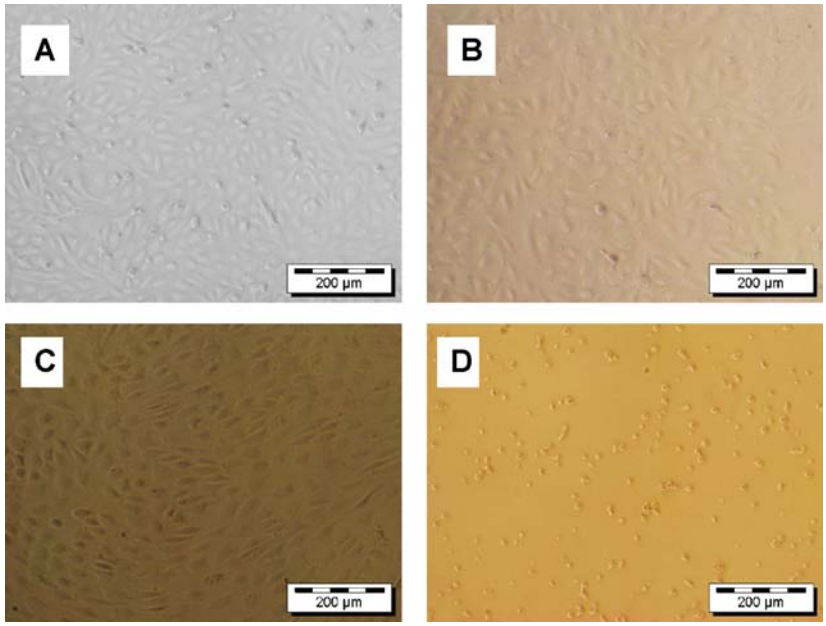

Fig. 1 Phase contrast micrographs of EC cultures. A: HSVECs; B: CD 34+ derived ECs; C: CD133+ derived ECs 30 days old; D: CD133+ derived ECs 4 days old

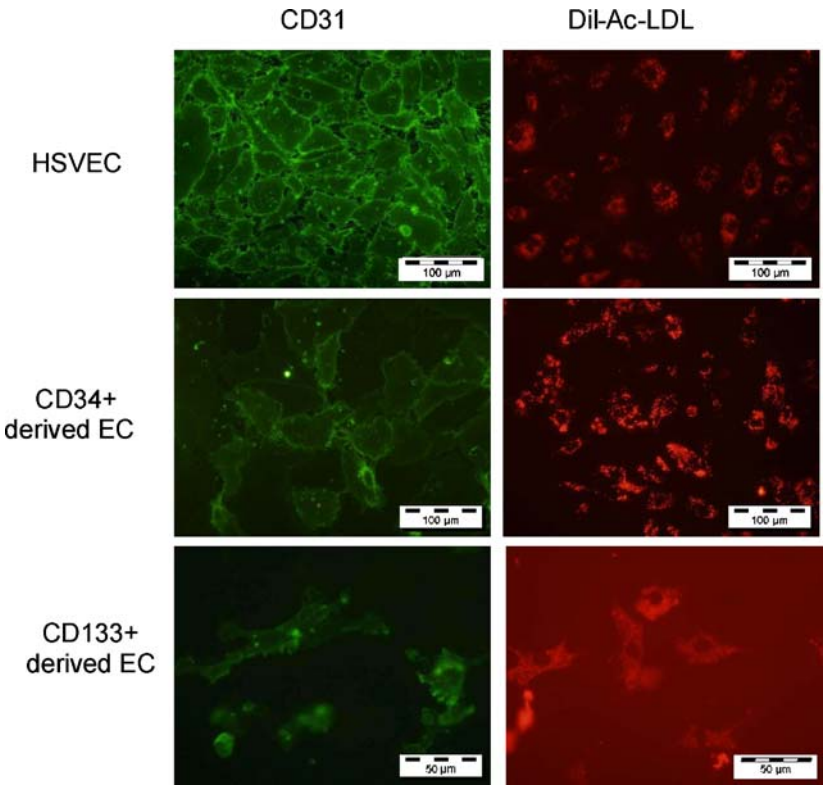

Fig. 2 Fluorescence images of CD31 staining and acetylated low-density lipoprotein (Dil-Ac-LDL) uptake in HSVECs, CD34+ derived CEs and 30 days old CD133+ derived ECs

incorporated Dil-Ac-LDL (Fig. 2). Reverse transcriptase-PCR of total RNA extracted from CD133+ derived ECs (lanes 1-3 from 3 different donors) and HSVECs (pool in lane 4), demonstrated the presence of KDR, CD31 and VE-cadherin whatever the cell origin (Fig. 3).

Using the described method to prepare pullulan hydrogels, it was possible to obtain homogeneous, transparent and easy to handle gel discs. No weight loss or degradation of the scaffold was evidenced upon storage in sterile PBS at 4°C for up to 4 weeks.

The attachment kinetics of ECs on scaffolds is presented in Fig. 4(A) and (B).

Previous experiments have established that endothelial cell counting could be quantified by absorbance measurement with a direct evident relationship between the number of cell and N-acetyl-β-D-hexosaminidase enzymatic activity

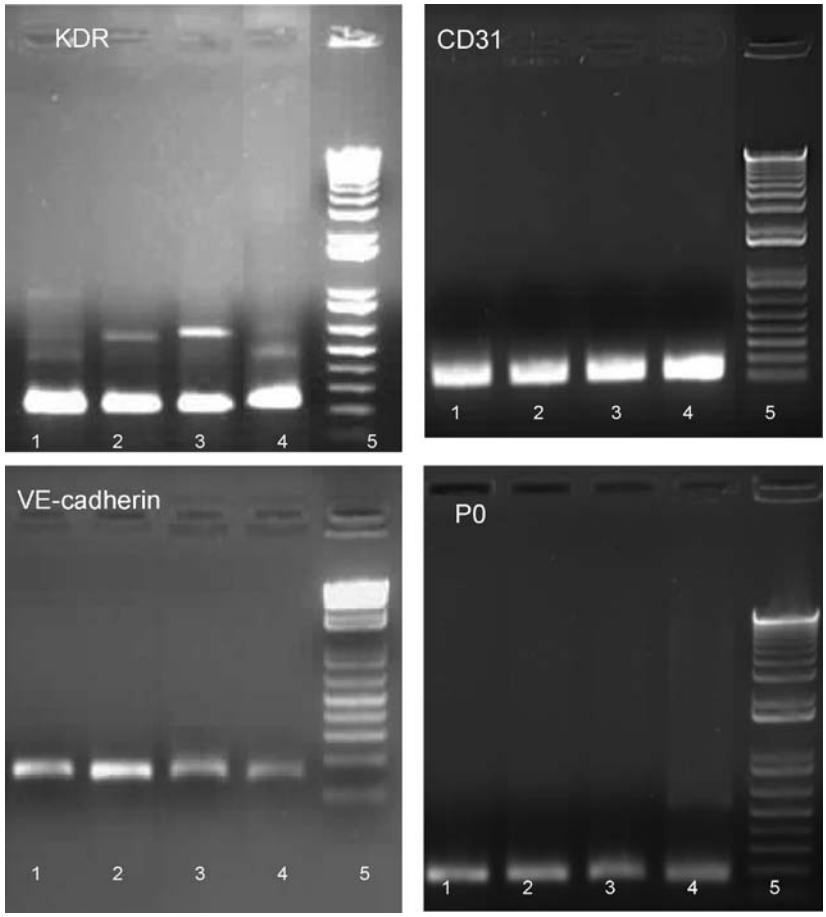

Fig. 3 Reverse transcriptase-PCR showing the KDR, CD31, VE-cadherin and Po (housekeeping gene) mRNA of HSVECs and 30 days old CD133+ derived ECs: lanes 1–3 refer to 3 different donors of bone marrow, 4 refer to a pool of HSVECs and lane 5 to markers

(data non shown). An overall comparable pattern at 4 and 24 h was observed, that is the highest values for 30d CD133+ derived ECs followed by CD 34+ derived cells significantly different on both surfaces when compared with HSVECs, and a cell adhesion significantly inferior (for HSVECs, 30d old CD133+ derived ECs, CD 34+ derived ECs) or identical (4d old CD133+ derived ECs) on hydrogels than on controls. No significant differences were obtained between hydrogels coated or not with 0.1% gelatin (data not shown).

4 Discussion

Since 1997, it is known that a cell population called endothelial progenitor cells can be isolated from circulating mononuclear cells [16, 17], bone marrow [18] and cord blood [19, 20]. Laboratory evidence suggests that these cells express a number of endothelial-specific cell-surface markers and exhibit numerous endothelial properties. Kaushal et al. [21] have published the first animal implantation of *in vitro*-lined ePTFE grafts with ECs derived from *in vitro* differentiation of precursor cells present in circulating peripheral blood. More recently, Yang et al. [22] lined polyurethane grafts with human EPCs and implanted them in dogs. Adult bone marrow contains at least two types of stem cells: hematopoietic stem cells and mesenchymal stem cells. Among the former is the presence of "hemangioblast" precursors of both hematopoietic and endothelial cells [23]. Such hemangioblasts (HMBs), found in bone marrow, express hematopoietic stem-cell markers like CD34 or CD133, as well as the endothelial marker VEGFR2. These cells are released from the bone marrow to the peripheral blood upon mobilization with cytokines, synthesized by ischemic tissues or tumors. The mobilized cells then travel to the ischemic tissues or tumors and can give rise to endothelial vessels and contribute to new capillary formation. The bone marrow also contains mesenchymal stem cells (MSCs), which have been shown to develop into endothelial cells [24] and improve vascularisation *in vivo*. MSCs can also be mobilized to the peripheral blood upon ischemia and contribute to tissue repair [25]. As a new source of endothelial cells, proliferating human endothelial progenitor cells isolated from adult tissues can be used to engineer artificial vessels, repair damaged vessels, and to induce the formation of vessel networks in engineered tissues [25–27]. Stem cells derived from adults have great therapeutic potential, but much research is still needed before their clinical use becomes commonplace [28].

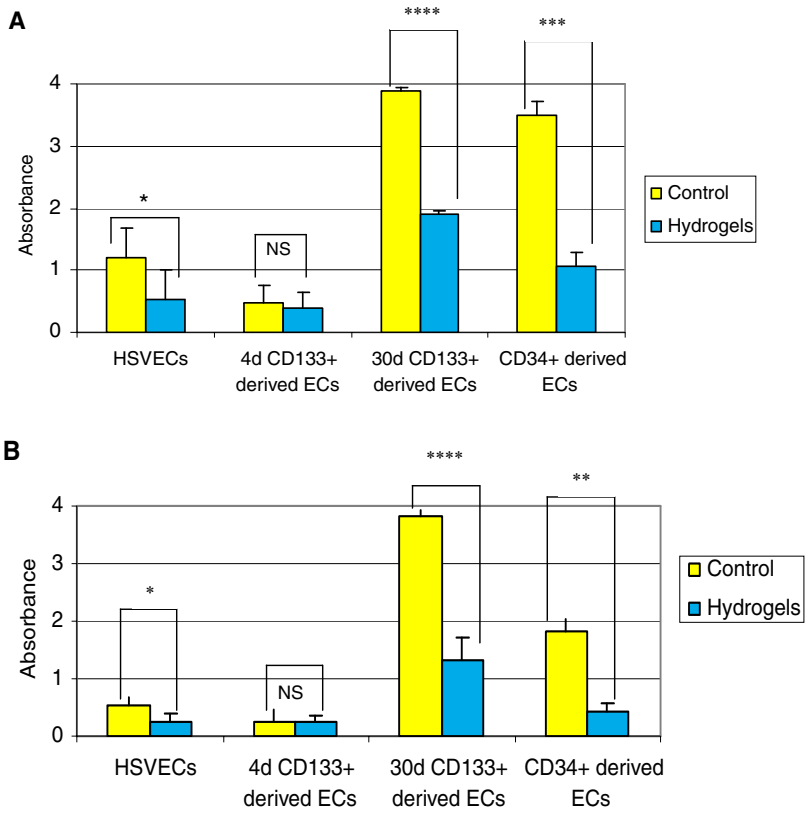

Fig. 4 Representative histograms of cell adhesion (A: 4 h, B: 24 h) on hydrogels for 4 cell types performed as described and expressed in absorbance unit: 4 cell types seeded on cultures plates. $*p < 0.01$; $**p < 0.02$; $***p < 0.05$; $****p < 0.001$

Very few studies have reported the isolation of EPCs from human bone marrow [4, 5]. From our results it seems that cells with an endothelial phenotype could be derived from CD133+ one month after cell isolation. Indeed, endothelial cell markers commonly used for characterisation were positive. Although, Liew et al. published in their review article that EPCs from peripheral blood consist of two different subpopulations (early and late EPCs) [29] with distinct growth patterns and ability in secretion, it was not possible from our results to answer such an issue.

To our knowledge, this study is the first to use CD133+ derived ECs for cytocompatibility to provided vascular tissue which could be used for engineering purposes.

We describe here the synthesis and characterisation of novel biocompatible hydrogels prepared by cross-linking polysaccharides as well as the possibility of culturing endothelial cells on these gels. A cross-linking process carried out in aqueous conditions provided a simple method to obtain a polysaccharide-based scaffold [30, 31]. This type of hydrogel is particularly suitable for *in vitro* studies since it is easily handled, and can be molded or cut to the desired shape and thickness. Moreover, it is transparent which allows for direct light microscopic observations of the seeded cells. No cytotoxicity of this cross-linked pullulan hydrogel was evidenced whereas some dextran/hyaluronan hydrogels formed in the presence of photoinitiators and ultraviolet radiation were shown to affect vascular smooth muscle cell proliferation [32].

The results of these experiments suggest that EPCs can exhibit required high levels of adhesion on hydrogels in serum-free conditions, a pre requisite for vascular tissue engineering. Further experiments are in progress for the subsequent cytocompatibility evaluation that is cell proliferation, expression of differentiated phenotype and cell behaviour under controlled shear stress conditions.

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