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Microarchitecture of poly(lactic acid) membranes with an interconnected network of macropores and micropores influences cell behavior

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ABSTRACT

Tissue engineering scaffolds require a three-dimensional architecture with controlled pore size and structure to host tissue formation. Here we used the non-solvent induced phase separation (NIPS) process to prepare porous asymmetric membranes made of poly(lactic acid) (PLA) using dimethylformamide (DMF) as solvent and water as non-solvent. The addition of a polymer additive to the PLA solution has made it possible to increase and control the size of open macropores at the membrane bottom surface and to generate a dual pore architecture consisting of an interconnected network of macropores and micropores. Based on the study of the compatibility of PLA with the polymer additive we propose that the dual pore architecture is generated through a two phase separations process. Membrane surface could be functionalized with hyaluronan (HA) and, whatever the cell type considered, HA-functionalization enhanced cell proliferation. Interestingly, a strong effect of macropore size and surface functionalization by HA on cell colonization and cell proliferation within membranes and membrane morphology on cell organization was demonstrated. Cancer cell lines were able to adopt an *in vivo* tumor-like behavior, endothelial cells reconstituted a vascular-like structure and mesenchymal stem cells adopted a specific organization which can open perspectives for their use and conservation. These results show that our membranes with controllable dual pore architecture offer great potentialities in tissue and tumor engineering.

1. Introduction

Tissue engineering applications commonly require the use of three-dimensional (3D) scaffolds to provide a suitable microenvironment for the incorporation of cells to regenerate damaged tissues or organs. Scaffolds serve to mimic the *in vivo* microenvironment where cells interact and behave according to the biological and the mechanical cues obtained from the surrounding environment [1–3]. These scaffolds are generally porous biomaterials designed to promote cell-cell, cell-biomaterial interactions and extracellular matrix deposition and to allow transport of gases, nutriments and soluble factors as well as waste removal. To meet these requirements, 3D scaffolds must have a network of interconnected macropores and micropores. Cell behavior is directly affected by the scaffold architecture since the extracellular matrix provides cues that influence the specific integrin–ligand interactions between cells and the surrounding [4]. Hence, cell proliferation, organization and differentiation rely on the scaffold spatial properties such as porosity and pore size [5]. Although porosity is needed, the ratio between the surface and the volume of the scaffold should not be too great to maintain its mechanical strength [6,7]. The pore size has been shown to influence cell alignment, orientation [8], multicellular organization [9], cell spreading [10] and cell attachment [11]. However, there are conflicting reports on the optimal pore size which means that the relationship between pore size and cell behavior is still poorly understood [4].

Porous scaffolds made of polymers have been developed using various techniques [6,12,13] among which phase separation, particulate leaching, gas foaming, freeze-drying, hydrogel, electrospinning, micro-molding, 3D printing, stereolithography, selective laser sintering and fuse deposition modeling. Phase separation processes can be

Abbreviations: CD44, cluster of differentiation 44; DMF, dimethylformamide; DSC, differential scanning calorimetry; HA, hyaluronan; HDA, hexane-1,6-diamine; HMEC-1, human microvascular endothelial cell; MSC, mesenchymal stem cell; NIPS, non-solvent induced phase separation; NMP, 1-methyl-2-pyrrolidone; PBS, phosphate buffer saline; PEG, polyethylene glycol; PLA, poly(lactic acid); PSf, polysulfone; PVP, polyvinylpyrrolidone; RHAMM, receptor for hyaluronan-mediated motility; SEM, scanning electron microscopy; TIPS, thermally induced phase separation; VIPS, vapor induced phase separation

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classified into non-solvent induced phase separation (NIPS), thermally-induced phase separation (TIPS), and vapor induced phase separation (VIPS) [14,15]. These processes have been widely used for a long time to develop polymeric porous membranes for a variety of industrial applications such as microfiltration, ultrafiltration [16], reverse osmosis, dialysis [17] and gas separation [15]. Since phase separation methods enabled the generation of porous polymeric materials, they were used to prepare 3D scaffolds for tissue engineering. Various attempts have been made to develop scaffolds by the NIPS process. However, even though the obtained scaffolds showed a 3D porous architecture [18] and allowed cell adhesion and proliferation [19-24], they usually had pore diameters ranging from nanometer to a few tens of micrometers which is often too small to allow the cells to penetrate inside [13,19,22,25]. To overcome this problem, the NIPS process has thus been combined with other techniques such as micro-molding [26–28] or gas foaming [29]. The use of TIPS processes has been more effective in preparing polymeric scaffolds with interconnected macropores [30–33]. Therefore, the NIPS process has been much less used than the TIPS one to prepare scaffolds for cell culture. However, the NIPS process has the advantage of being simple and inexpensive especially from the energy point of view [14].

The aim of the present work was to use the NIPS process to prepare 3D porous scaffolds for tissue engineering in the form of asymmetric membranes with a nanoporous face allowing the diffusion of soluble compounds and gases and a macroporous face allowing the cells to penetrate inside the scaffold. Usually asymmetric membranes prepared by the NIPS process consist in a thin and dense top layer supported by a porous sublayer that often contain large void spaces [34]. To improve the separation performance and mechanical strength of the membranes used for various separation processes, much work has been done in recent years in order to remove macropores in favor of a micropore network [21,34–39]. Contrary to what was done for these separation membranes, the objective here was to obtain membranes with sufficiently large void spaces, or macropores, which are open on the lower face of the membrane. Such asymmetric membranes may constitute interesting 3D scaffolds for the cell culture and the study of cell behaviors *in vitro* as well as for tissue engineering, in particular, for applications where it is important both that the cells are in contact with the tissue to be restored and that they cannot spread elsewhere in the body.

Poly(lactic acid) (PLA) was chosen to prepare the asymmetric membranes. Indeed, PLA shows several advantages with respect to biomedical applications: it is biocompatible and biodegradable, it can be easily processed and it has good mechanical properties [40]. PLA is already used in a wide variety of applications such as sutures, drug delivery vehicles, prostheses, vascular grafts, bone screws, tissue engineering scaffolds and pins for fixation [40,41]. Although PLA scaffolds are known to be biocompatible and to have better mechanical properties than those made of natural polymers, due to the hydrophobicity of PLA, they have a rather weak cytocompatibility. To overcome this problem, various methods have been used to chemically modify the surface of PLA scaffolds [42]. In our case, we chose to functionalize the surface of our PLA scaffolds previously aminolyzed with hyaluronan (HA). Indeed, HA is a non-adhesive glycosaminoglycan which has the main advantages of being a major constituent of the extracellular matrix of human tissues and more generally of vertebrate tissues, of being non-immunogenic and of being commercially available in biomedical quality [43]. HA is also known for its biological and structural functions [43,44].

In the present paper, we show how by choosing a good solvent – non solvent system and using a polymer additive as porogen, we were able to use the NIPS process to prepare porous asymmetric membranes of PLA with the desired characteristics to make them 3D scaffolds for tissue engineering. These membranes showed a high porosity, a nanoporous top surface and a bottom surface with macropores open to the surface whose diameter was large enough to allow the cells to enter into the scaffold and colonize it. Indeed, an original interconnected network of macropores and micropores was also observable inside the asymmetric membranes. To explain the origin of this new architecture inside membranes prepared by the NIPS process we propose a two phase separations process: polymer/polymer and polymer/solvent. By selecting the appropriate experimental parameters, we prepared a range of PLA asymmetric membranes with different mean diameters of macropores open to the surface. We investigated the role of macropores size on cell proliferation and organization of several normal and cancer cells types cultured in our asymmetric PLA membranes. We showed that, although the functionalization of the surface of our scaffolds with HA clearly improved their cytocompatibility, our scaffolds had a porous architecture particularly well-suited to the development of eukaryotic cells and thus they offer interesting potentialities in tissue engineering.

2. Materials and methods

2.1. Preparation and characterization of PLA membranes

2.1.1. Preparation of asymmetric PLA membranes

Asymmetric PLA membranes were prepared according to the NIPS process. PLA granules were supplied by NaturePlast (France). PLA, of PLE 005 grade, contained 4% D- and 96% L-enantiomers. Its mass-average molar mass (M_w) and its number-average molar mass (M_n) equal to $145{\cdot}10^3$ $g{\cdot}mol^{-1}$ and $60{\cdot}10^3$ $g{\cdot}mol^{-1}$ respectively were determined by high performance size exclusion chromatography – multi-angle laser-light scattering – refractive index. Polyethylene glycol (PEG) of various molar masses (3400, 8000 and 20,000 $g{\cdot}mol^{-1}$) and polyvinylpyrrolidone (PVP) with a molar mass of 10,000 $g{\cdot}mol^{-1}$ from Sigma Aldrich (France) were used as porogen. Dimethylformamide (DMF) (Merck, France) was used as polymer solvent whereas water (Milli-Q water, resistivity of 18 $\Omega{\cdot}cm^{-1}$, Millipore) was used as PLA non-solvent. Polymer solutions were obtained by dissolving PLA and the porogen polymer in appropriate mass ratios in DMF at 70 °C under a dry atmosphere for about 2 h to ensure their complete dissolution. After bubble elimination, the cooled solutions were cast onto a glass plate and spread using a Gardner knife to form films approximately 250 µm thick. The glass plate carrying the polymer film was then immediately immersed in a Milli-Q water bath at room temperature (i.e. 20–22 °C). After coagulation, the formed membrane was thoroughly washed with Milli-Q water and stored in a desiccator under vacuum at room temperature. Membranes thus prepared have a thickness of 160 ± 2 µm. Membrane thickness was measured in 20 points for each membrane using a Palmer micrometer.

2.1.2. Preparation of polymer films by solvent evaporation

PLA, PEG, PVP, PLA-PEG and PLA-PVP solid films were prepared by solvent evaporation. PLA, PEG and PVP films were prepared from a 7% (w/w) polymer solution in DMF. PLA-PEG and PLA-PVP films were prepared from a solution containing 7% (w/w) PLA and 7% (w/w) of either PEG (8000 $g{\cdot}mol^{-1}$) or PVP (10,000 $g{\cdot}mol^{-1}$) in DMF. For each film, a volume of the polymer solution was deposited in a glass petri dish. The dishes were then placed on a hot plate at 60 °C until the solvent evaporated.

2.1.3. Structural and morphological characterization

Each face of membrane samples was observed by optical microscopy (Leica DMLM). They were also observed by scanning electron microscopy (SEM) (Zeiss EVO40). In order to avoid large changes in the membrane shape and morphology, the interior structure of the membranes was observed using membrane samples which were dipped in liquid nitrogen before they were fractured. The mean pore diameter of pores open on each face of the membrane sample was determined by averaging the diameters of 20 pores measured using the Sesame software (Microvision Instruments). For the lower face, this was done from both optical microscopy and SEM images, while for the top face, this was done only from SEM images.

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