Surface Patterning and Biological Evaluation of Semi-Interpenetrated Poly(HEMA)/Poly(Alkyl β-malolactonate)s

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Summary: Semi-interpenetrating hydrogel networks were prepared by radical polimerization of monomer HEMA, co-monomer EGDMA as cross-linking agent and in the presence of 1 wt % of poly(alkyl β-malolactonate)s. Biological evaluation in terms of cell adhesion and proliferation of the prepared materials showed the ability of HEMA-based hydrogels to sustain a good cell adhesion and proliferation. Moreover, a method based on soft-lithography to obtained surface microstructured semi-interpenetrating hydrogel networks was developed, thus allowing for further investigation of the influence of surface topography on cell behavior.

Keywords: hydrogels; 3T3 mouse embryo fibroblast; poly(alkyl β-malolactonate)s; soft-lithography; tissue engineering

Introduction

Tissue engineering is a relatively new and emerging interdisciplinary field that is applying the technology of bioengineering, life science, and clinical sciences for trying to solve critical medical problems of tissue loss and organ failure. The essence of tissue engineering is the use of cells together with either natural or synthetic extracellular components, in the development of implantable parts or devices for the restoration or replacement of tissue/organ. [1]

A critical issue in tissue engineering is represented by the development of new biologically compatible materials to be used as scaffolds that serve as permissive substrates for cell growth,

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differentiation, and biological functions. The ideal biocompatible scaffold should meet several criteria such as non cytotoxicity and the ability to promote cell-substrate interactions. Moreover, the basic units of such materials should be amenable to design and realize modifications, in order to meet specific needs and to obtain controlled biodegradation rates, as well as being in first instance chemically compatible with aqueous solutions and physiological environments. [2]

Among the different classes of biomaterials, hydrogels are characterized by unique properties such as low interfacial tension with body fluids and hydrodynamic and mechanical properties similar to those of soft tissues. The biocompatibility of hydrogels is due to their ability to simulate natural tissues, mainly for their high water content, surface softness, and high permeability to small molecules such as oxygen and metabolites. However, their low interfacial tension of hydrogels does not allow for surface deposition of serum proteins, whereas cell adhesion onto a polymer surface is mediated by pre-coating with serum proteins.

Moreover, natural tissues and organs are characterized by a very complex structure whose topography plays an important role in controlling cell adhesion and proliferation. [3,4] The ability to design and create biologically relevant patterns on biomaterial surfaces provides new capabilities for cell biology and tissue engineering. [5,6]

Recently, we started a research aimed at the accomplishing bio-functionalization of 2-hydroxyethyl methacrylate (HEMA) hydrogels in order to obtain scaffolds able to sustain cell adhesion and proliferation for tissue engineering application.

Attention was focused on two possible strategies. Indeed, chemical modification of HEMA based hydrogels may be performed by inclusion of biologically active polymers. This will promote the interactions of serum proteins with the hydrogel surface, resulting in the enhancement of cell adhesion and proliferation. On the other hand, the possibility of obtaining hydrogels with specific surface patterns by soft-lithography could not only modify biological interactions but also provide insight on the influence of surface topology on cell behavior.

Experimental

Alkyl β-malolactonate homopolymers and copolymers were prepared by following a procedure reported previously. [7]

Preparation of HEMA Based Semi-Interpenetrating Networks

Semi-interpenetrating network based on poly(2-hydroxyethyl methacrylate) (HEMA) and poly(alkyl β-malolactonate)s were prepared by the polymerization of a mixture containing HEMA, 3 wt % of ethylene glycol dimethacrylate (EGDMA), and 1 wt % of poly(alkyl β-malolactonate). The reactions were carried out at 37 °C for 4 h in presence of 0.75 % of the redox couple ammonium persulfate/sodium metabisulfite. [8]

Biological Evaluation of HEMA Based Semi-Interpenetrating Networks

Hydrogel disks with appropriate dimensions to fit in the wells of tissue culture polystyrene plates, were immersed in pH 7.4 phosphate buffer saline (PBS) for 24 h and then UV sterilized for 15-30 min. The 3T3 balb/c mouse fibroblast cells were seeded at an appropriate density on the hydrogel films and checked for adhesion 2 h later. The culture medium was then removed and replaced with fresh culture medium. In cell proliferation assays, cells were incubated on hydrogel films up to 72 h after seeding. Quantitative proliferation assays were performed by using the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Germany) in accordance to the protocol indicated by the manufacturer.

Preparation of Patterned Silicon Wafers

After layout the desired patterns using computer aided design, the CAD file was transferred to a pattern generator. A blank mask was put into pattern generator and was exposed as the design. Then, the mask was developed and the chrome was etched. Finally, the resist was stripped. After rinsing and blow dry, the photomask with designed pattern was ready to use.

A silicon wafer was primed with P-20 (20% HMDS) primer. OiR 897-12i was spun at 4000rpm for 30 seconds. Post bake at 90°C for 60 seconds on a hot plate was then applied and followed by exposure at 365nm for 2.4 seconds, and finally baked at 115°C for 60 seconds before the final development in CD-26 for 60 seconds.

Wafer patterning was released by etch treatment in a PlasmaTherm SLR-770 Chlorine Etcher for certain amount of time depending on the desired pattern depth. The wafer then was soaked in Shipley 1165 remover for photoresist stripping and after rinsing and blow dry, the patterned silicon wafer was ready to use.

Preparation of Surface Patterned HEMA Hydrogels

Hydrogels with microstructured surface were prepared by polymerization of a mixture of HEMA, 3 wt % of EGDMA, and 0.75 wt % of sodium metabisulfite/ammonium persulfate at 37 °C for 4 h. The monomer mixtures was polymerized directly onto patterned silicon wafers previously primed by soaking in 1 % solution of 1H, 1H, 2H, 2H-perfluorooctylthriclorosilane in dichloromethane for 22 h.

Results and Discussion

Preparation of HEMA based semi-interpenetrating networks

The goal to obtain materials that combine the good biocompatibility of HEMA based hydrogels with the ability of poly(alkyl β-malolactonate)s to trigger cell adhesion and proliferation [9] was pursued by polymerization of HEMA/EGDMA mixtures containing preformed poly(alkyl β-malolactonate)s. This method led to the formation of a semi-interpenetrating network (semi-IPN) in which the poly(ester) component was physically entangled within the crosslinked HEMA hydrogels (Table 1).

The synthesis of the selected poly(β-malolactone)s, namely poly(β-malolactone) (pML), poly(benzyl β-malolactone) (pBzML), and a 20:80 copolymer of cholesteryl β-malolactone/benzyl β-malolactone (ChML20) has been reported elsewhere [7]. The polymerization mixtures consisted of HEMA, 3 wt % of EGDMA, 1 wt % of poly(alkyl β-lactonate), and 0.75 % of a 1:1 solution of ammonium persulfate/sodium metabisulfite as redox initiator. The feed mixture was injected between two silanized glass plates separated by 100 μm thick silicon spacer and cured at 37 °C for 4 hours. The recovered films were washed with

distilled water and then incubated in 0.01 M pH 7.4 phosphate buffer (PBS) for 24 hours at 37 °C. This procedure allowed for the purification of hydrogels from low molecular weight compounds possibly present within the matrix. Films were then dried over 96 % sulfuric acid solution until constant weight was reached. The hydrophilicity of the prepared semi-IPN's was evaluated by soaking the polymer films in PBS at 37 °C.

It is well known that the hydrophilic/hydrophobic balance of biomaterials has an important influence on cell response, in terms of both adhesion and proliferation. Indeed the presence of even small amounts of poly(alkyl β-malolactonate)s in HEMA hydrogels provided a small but significant modification of their swelling degree. In particular, the presence of the hydrophobic poly(benzyl β-malolactonate) gave rise to a decrease in the swelling degree from 50 to 47 % whereas the hydrophilic poly(β-malolactone) determined an increase from 50 to 53 % (Table 1).

Table 1. Poly(alkyl β-malolactonate)s used in the preparation of HEMA based semi-IPNa

Polymer sample	Poly(alkyl β-lactonate)		
	Alkyl group	Mw	Swelling[b] (%)
E3/pML	COOH	-	53
E3/BzML1	benzyl	20900	47
E3/BzML2	benzyl	18000	48.3
E3/ChML20	benzyl/cholesteryl	6500	44

[a] The polymerization mixture contained also 1.0 g of HEMA, 30 mg of EGDMA, 38 μl of a 0.526 M water solution of sodium metabisulfite and 38 μl of a 0.438 M water solution of ammonium persulfate; runs were carried out at 37 °C for 4 h. [b] Swelling degree measurements carried out in Phosphate Buffer Saline at 37 °C until constant

Biological Evaluation of HEMA Based Semi-Interpenetrating Networks

Biological investigations were carried out on 100 μm thick hydrogels. After purification in PBS at 37 °C for 24 hours, films were carefully cut into a proper shape to fit the well of tissue culture poly(styrene) plates. Particular attention was devoted to a complete covering of the bottom and the sides of the well, so that no cell would come into contact with poly(styrene). Films were sterilized by UV irradiation for 15-30 minutes and then equilibrated in Dulbecco's Modified Eagles Medium (DMEM) at 37 °C for at least 6 hours. It is important to notice that DMEM did

not contain serum, in order to avoid the possible deposition of proteins on the hydrogel surface before cell seeding. The 3T3 balb/c mouse fibroblast cell line, typically used in cytotoxicity and cytocompatibility investigations, was seeded at a density of 3 x 10^4 cells/cm^2. After a 72 hours incubation, samples were analyzed with WST-1 proliferation reagent for a quantitative evaluation of cell proliferation. Additionally, after the cells were fixed and stained, the films were removed from the wells and analyzed under the optical microscope for morphology characterization. An HEMA/EGDMA hydrogel film was used as control. Very limited adhesion and proliferation was possible to be observed on the control sample (Figure 1a). However, cells did not reach confluence during the experiment lifetime, showing a tendency to grow in a net fashion. Nevertheless, some cell adhesion and proliferation occurred. This behavior can be attributed to the presence of EGDMA, a fairly hydrophobic crosslinking agent, which may have introduced hydrophobic spots on the hydrogel surface allowing for cell adhesion. Experiments carried out on semi-interpenetrating networks prepared with HEMA, 3 wt % EGDMA, and 1 wt % poly(alkyl β-malolactonate) showed good results when pBzML (Figure 1b) and cChML 20 (Figure 1c) were used as the poly(ester) component. In contrast, samples containing 1 wt % of poly(ß-malolactonate), a strongly hydrophilic polymer, supported limited 3T3 cell proliferation. Moreover, cells formed small islets on the hydrogel surface and displayed a peculiar morphology, that was extremely small and round shaped (Figure 1d). This result seems to confirm that the presence of hydrophilic polymers does not allow for proper cell proliferation.

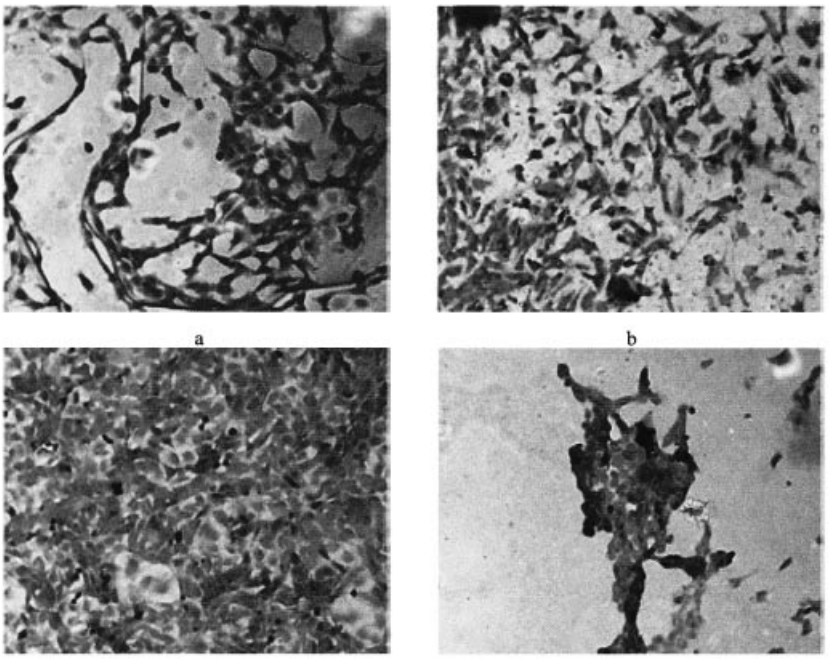

Figure 1. Optical micrograph of 3T3 cells grown on HEMA–poly(alkyl β-malolactonate)s semi-interpenetrating network; a) pHEMA; b) pHEMA/pBzML, c) pHEMA/cChML20; d) pHEMA/pML.

Preparation of Surface Patterned HEMA Hydrogels

2-Hydroxyethyl methacrylate (HEMA) based hydrogels with microstructured surface were prepared by polymerization of the monomer mixtures directly onto patterned silicon wafers (Figure 2).

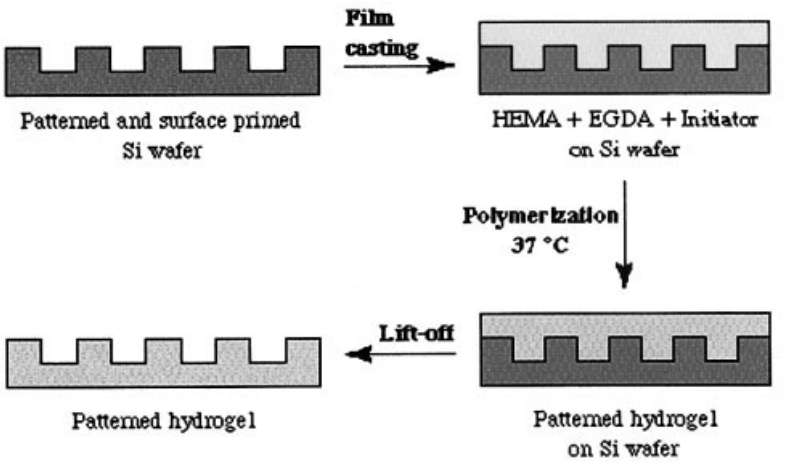

Figure 2. Schematic representation of the procedure adopted for hydrogel patterning by soft lithography.

The pattern design consisted of twenty-five square patterns with lines of 1, 2, 4, 8, and 16 μm width and spacing. The depth of patterns was 3, 5, and 10 μm depending on the etching time. The adopted design and process, allowed the creation of trenches and ridges with size scale comparable to that of many biological systems and for investigating the role of surface topography to cell adhesion, growth, and differentiation.

Hydrogels were prepared by radical polymerization of HEMA/EGDMA mixtures in the presence of sodium metabisulfite/ammonium persulfate redox initiator, at 37 °C for 4 hours directly loaded on the silicon wafer.

Lift-off of the polymerized hydrogel from the silicon wafer represented a major issue, as the applied mechanical stress modified the original shape of patterns. To overcome this problem, the silicon wafer surface was changed by soaking the wafer into a 1 % solution of 1H, 1H, 2H, 2H-perfluorooctyltrichlorosilane, a strongly hydrophobic compound, in dichloromethane for 12 and 22 hours. Better results were obtained when wafers submitted to a longer time were used. The primed silicon wafer was attached on a silanized glass plate and covered with a second silanized glass plate separated by 1 mm thick silicon spacer. Then a mixture of HEMA, 3 wt % of ethylene

glycol dimethacrylate (EGDMA) as crosslinking agent, and the redox initiator was injected between the two glass plates containing the silicon wafer and cured at 37 °C for 4 hours. At the end of the polymerization, the glass in direct contact with the polymerized hydrogel was removed and hydrogel was soaked in water for at least 12 hours. The strain connected to stress imposed by the swelling process, combined with the highly hydrophobic surface of the primed silicon wafer, made possible the lift off of the surface patterned hydrogel without the application of even a minor mechanical stress.

Characterization of Surface Patterned Hydrogels

Surface patterned hydrogels were analyzed by optical microscopy either in the dry state or in the swollen state. This technique allowed for verifying that the surface pattern was still preserved after a substantial number of swelling/drying cycles (Figure 3). However, careful investigation of the dimensional variation in going from the dry state to the swollen state is beyond the confidence limits of optical microscopy.

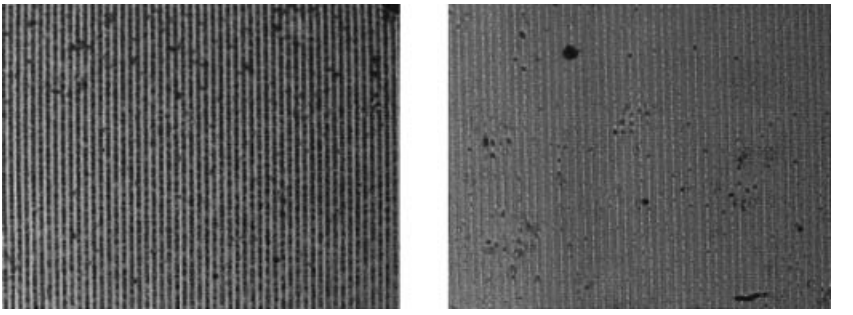

Figure 3. Optical micrograph (10X) of dry (left) and swollen (right) surface patterned HEMA hydrogels.

Surface patterned HEMA hydrogels were analyzed in the dry state by scanning electron microscopy (SEM). This technique gave a better picture of the dimensions and of the morphology of the pattern microstructure as compared to optical microscopy. However,

exposure of the hydrogel to the electron beam caused the deterioration of the soft material during the analysis, which somewhat altered the pattern morphology.

A more accurate analysis of dry patterned hydrogels was carried out by atomic force microscopy (AFM) (Figures 4). The results of this investigation indicated that grooves in the hydrogel are always smaller than those printed on the silicon wafer master. Nevertheless, the overall dimensions are very well preserved, although the edges of the ridges were round shaped rather than squared, and the surface was usually rough.

Patterns containing 1 μm size of the groove and/or of the ridge showed a swelling-deswelling cycle morphology collapse. This result indicates that the hydrogel structure may not be strong enough to preserve features of sub-micron size. It is also possible that the hydrophobicity of the primed wafer prevented penetration of the rather hydrophilic monomer mixture into the narrowest grooves. Shrinkage of the feed mixture during the polymerization process may also be responsible of the observed behavior. Investigations aimed at clarifying this point are currently in progress.

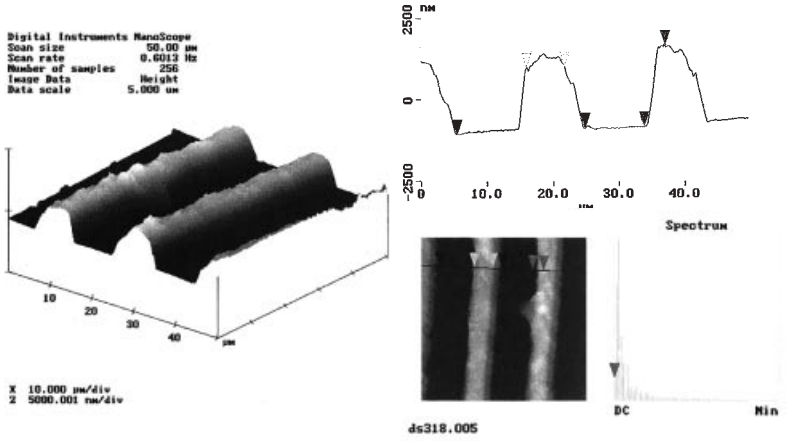

Figure 4. AFM pictures of dry hydrogel pattern (16 μm grooves and 4 μm ridges): 3D image (left) and section analysis (right).

Conclusions

Bio-functionalized HEMA hydrogels were prepared by polymerization of HEMA in a solution of preformed poly(alkyl β-malolactonate)s. Analysis of the swelling degree of the networks containing 1 wt % of poly(alkyl β-malolactonate)s clearly demonstrated that even such a small amount can significantly affect the swelling behavior of the hydrogels.

Semi-interpenetrating networks prepared by physical inclusion of poly(alkyl β-malolactonate)s within HEMA hydrogels were able to sustain and promote cell adhesion and proliferation; the most hydrophobic and propensive to selfstructuring poly(alkyl β-malolactonate) (cChML20) gave the best results. None of the investigated materials exhibited toxic effects. In spite of the preliminary character of this investigation, the lactonate-modified hydrogels displayed promising characteristics that could be exploited in tissue engineering technology.

Adoption of soft lithography allowed for the straightforward preparation of surface patterned HEMA hydrogels. By using this technique, 1-16 μm ridges and grooves were imprinted on the hydrogel surface. Atomic force microscopy showed that features larger than 1 μm were preserved through water swelling-drying cycles. By taking into account that the typical size of human cells is several micrometers, this technology seems promising for the preparation of micromachined polymeric scaffolds to reproduce the complex intricacies specific of living tissues.

Acknowlegments

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