Poly(ethylene oxide) Macromonomer Based Hydrogels as a Template for the Culture of Hepatocytes

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Summary: Macromonomer based poly(ethylene oxide) (PEO) hydrogels were tested with respect to their ability to serve as a template for the survival and the growth of hepatocytes. Two systems were considered : either the surface of pre-existing hydrogels, with controlled structural parameters, were seeded with isolated rat hepatocytes or the hepatocytes were dispersed in physiological medium containing the macromonomer/initiator and heated to 37°C. In the first case, cells were examined at given times after spreading over two days. The results were compared to those observed for the dispersion of fibroblasts onto a surface of the same type of hydrogels. The effects of the structure of the hydrogels and its chemical nature on the extent of hepatocyte attachment (or encapsulation) and the morphology were investigated.

Keywords: biomaterials; crosslinking; hepatocyte encapsulation; hydrogels; polyethylene oxide

Introduction

Poly(ethylene oxide), (PEO) is an hydrophilic polymer which exhibits specific solution and solid state properties. Furthermore, the remarkable biocompatible properties of this polymer have already led to a wide number of biomedical applications[1-3]. Well-structured PEO hydrogels can be obtained directly in water or in physiological medium upon free radical homopolymerization of water-soluble bifunctional PEO macromonomers[4-7]. That approach combining polymerization in water and control in advance of the structural parameters of the resulting hydrogels presents several decisive advantages with respect to classical end-linking

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process or irradiation techniques. Such macromonomer based hydrogels served as semi-permeable biocompatible membranes for an artificial pancreas[4,6]. They have also been tested regarding their capacity to serve as a template for the growth of nervous cells[5].They may provide an interesting scaffold for cell adhesion and a three dimensional space for cell proliferation. After some considerations on the synthesis of the hydrogels, the major part of the work will be devoted to the ability of such PEO hydrogels to serve as a template for the survival and the growth of hepatocytes. Two systems will be considered : either the hepatocytes will be seeded on the surface of pre-existing hydrogels or they will be dispersed in physiological medium containing the macromonomer/initiator and heated to 37°C whereupon crosslinking is to be expected.

Results and discussion

Synthesis of the hydrogels

PEO macromonomers with number average molar masses equal to 6500, 11500 or 15000 $g.mol^{-1}$ and of controlled functionality were synthesized as described previously[4] and directly polymerized in water to hydrogels. Potassium peroxodisulfate or redox initiators were used as initiators (1 molar % versus double bond content) and the different reactions carried out at 37°C or 60°C. The gel point was reached between one and four hours dependent upon the temperature, the chain length of the macromonomer and /or the type of initiator. After preparation, the gels were placed in water for swelling. Once swollen to equilibrium, and free of linear non-connected chains, they were characterized in terms of their swelling behavior and uniaxial compression moduli according to procedures described in the literature. In most cases they were kept in water with 0.3 wt % sodium azide to avoid micro-organism proliferation. The physico-chemical characteristics of these PEO hydrogels have been given in several previous publications[4,6,7]. From these results, it can be concluded that PEO hydrogels over a large range of properties can be obtained by a rather simple procedure: direct polymerization of bifunctional macromonomers, easily accessible, in water solution. These properties can be controlled by the molar mass of the macromonomer, the concentration of the macromonomer and even the type of free radical polymerization initiator.

For identical preparation conditions and molar masses, we observed that with redox initiators, gel point is reached within one hour even at a temperature of 37°C. On the contrary, no crosslinking is noted when the reaction is conducted in the presence of potassium persulfate at 37°C. In that case a temperature around 60°C is a prerequisite to

achieve crosslinking. The mechanical properties, for a given molar mass of macromonomer precursor are lower for hydrogels obtained in presence of the redox initiators.

Table 1 . Experimental conditions for the synthesis and physico-chemical characteristics of some hydrogels obtained by homopolymerization of bifunctional PEO macromonomers.

Reference	Precursor Molar Mass [a)]	Macromonomer [b)]	ε(%) [c)]	Q_V (water)	E_g (water)	E_g (THF)	Q_v (THF)
A	6500	20%	3.6%	8.77	91300	-	-
B	11500	20%	4%	11.14	58300	62800	7.42
C	15000	20%	6.5%	16.54	22500	-	-

a)Number average molar mass of the PEO precusor chain expressed in $g.mol^{-1}$
b)Wt-% of macromonomer to be crosslinked
c)Amount of extractable polymer in wt. %
d)Q_{VTHF} and Q_{Vwater} are the volume equilibrium swelling degrees in THF and in water, respectively
e)$E_{G\ THF}$ and $E_{G\ water}$ are the uniaxial compression moduli in THF and in water, respectively, expressed in Pa

Preliminary studies made on hydrogels confirmed that dense networks i.e. materials characterized by high values of uniaxial compression moduli are not well suited for cell-culture. Therefore, among the different types of available hydrogels, we selected those obtained via redox initiators. They are less dense that networks resulting from polymerization of PEO macromonomers at 60°C with potassium persulfate.

Growth of hepatocytes on pre-existing PEO hydrogels

Series of recent studies showed the importance of the matrice nature for hepatocyte attachment and increasing longevity for rat hepatocytes cultured respectively in sandwich configuration and on Matrigel^R compared to collagen singles layers. These observations have been confirmed recently by L.Richert et al.[8]. Hepatocytes can also attach to surfaces on which sugar derivates have been immobilized. L.G. Griffth et al. [9] coupled sugar derivates to different types of radiation cross-linked poly(ethylene oxide) hydrogels.

In the present work unmodified PEO hydrogels, whose synthesis has been discussed above, were tested regarding their potentiality to serve as a template for the growth and survival of hepatocytes. Hepatocytes were isolated from rat liver by a modification of the collagenase digestion method[8]. Cell preparation with a viability higher than 85 % was used for the

further experiments. Surfaces of pre-existing hydrogels were seeded with isolated hepatocytes. Cells were examined at given times after spreading over two days. The effects of the structure of the surface of the hydrogels and its chemical nature on the extent of hepatocyte attachment and the morphology were investigated. The results were compared to those observed for the dispersion of fibroblasts onto a surface of the same type of hydrogels. Polystyrene (PS) was used as a reference surface. Hepatocytes attached rapidly onto the PS surface and form a monolayer. On the contrary, attachment was low on PEO hydrogel surfaces. The longer the precursor chains, the lower the attachment of the hepatocytes was. These results are coherent with observations made on PEO hydrogels seeded with fibroblasts[6]. The macromonomer based PEO hydrogels are constituted of PEO chains chemically crosslinked via small domains of hydrophobic polymethylmethacrylate units. Therefore some cells are also suspected to adsorb preferentially on these hydrophobic domains. This situation has yet to be clarified.

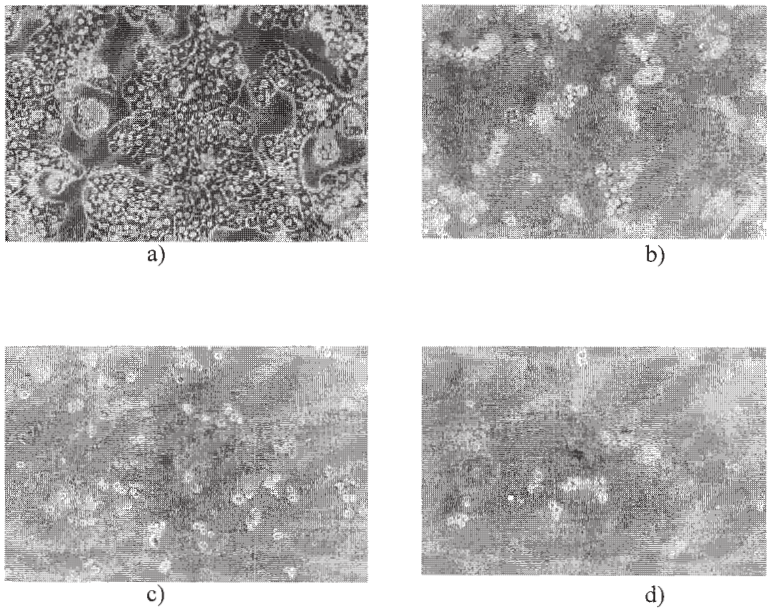
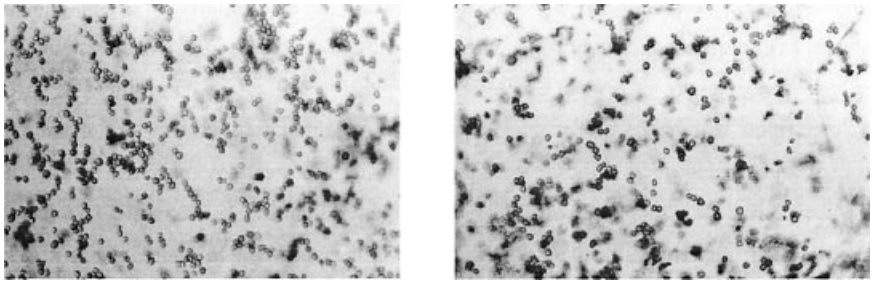


Figure 1 : Phase-contrast light micrographs of rats hepatocytes 24 h after seeding in various configurations (a) on a PS surface (b,c,d) on a PEO hydrogel of precursor molar mass (6500, 11500, 15000g.mol^{-1}) original modification X 50.

Encapsulation of cells during crosslinking

Surfaces of macromonomer based PEO hydrogels have just been shown to be well adapted for the growth of hepatocytes. The density of hepatocytes can be directly related to the nature of the surface and the crosslinking density of the hydrogel. In a previous publication[6], we showed that such macromonomer based hydrogels are also well suited as semi-permeable biocompatible membranes. Glucose and insulin diffuse through the material. These hydrogels may also be well designed as template for a tridimensional growth of hepatocytes into the material. To achieve that growth, hepatocytes have to be dispersed homogeneously in the gel. This is almost impossible for gels in the swollen state. One way to do it, could be to dry the gel and put it in a physiological medium containing the hepatocytes. Upon re-swelling hepatocytes should enter progressively the tridimensional structure. Incorporation of materials during re-swelling processes is a rather slow process and even over longer periods far from yielding homogeneous distribution of the hepatocytes. Earlier studies confirmed that bifunctional PEO macromonomers can be homopolymerized to hydrogels in less than one hour at 37°C. In addition, preliminary experiments confirmed the survival of the hepatocytes even in the presence of high concentrations of free radical polymerization initiators. This prompted us to proceed to crosslinking in the presence of isolated rat hepatocytes whereupon far more homogeneous dispersion of the hepatocytes should be reached. Fresh hepatocytes were dispersed in physiological medium containing the macromonomer precursor, the redox initiator and the solution was heated to 37°C. Two molar masses of precursor were selected 11 500 and 15 000 $g.mol^{-1}$ and the macromonomer weight concentration was by 20-wt %.



Precursor 11500$g.mol^{-1}$ Precursor 15 000 $g.mol^{-1}$

Figure 2 : Phase contrast light micrograph of rat hepatocytes encapsulated during free radical polymerization of bifunctional PEO macromonomers at 37°C after 1 hour in culture (magnification X50).

For purpose of comparison crosslinking was also performed on cell-free macromonomer solutions. In all cases crosslinking occurs generally after 30 to 60 min. Hydrogels free of encapsulated materials were purified and characterized according to usual procedures. Their properties are almost identical to hydrogels prepared earlier under similar conditions. No physico-chemical determinations could be made on the hydrogels containing the hepatocytes. In figure 2, we have presented the phase contrast light micrograph of hydrogels in which rat hepatocytes have been encapsulated. As it can be seen from these experiments, hepatocytes are dispersed in the hydrogels and are present at different levels in the gel. This is clearly revealed by the changes in coloration. These results could be further confirmed by figure 3, which shows the hepatocytes encapsulated in a PEO hydrogel (11500 $g.mol^{-1}$). The hepatocytes are dispersed in the hydrogel, but are much more concentrated at the bottom of the gel, due to the sedimentation of the cells during polymerization.

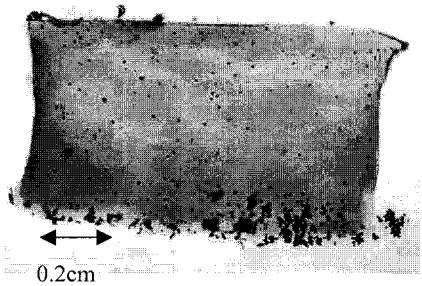
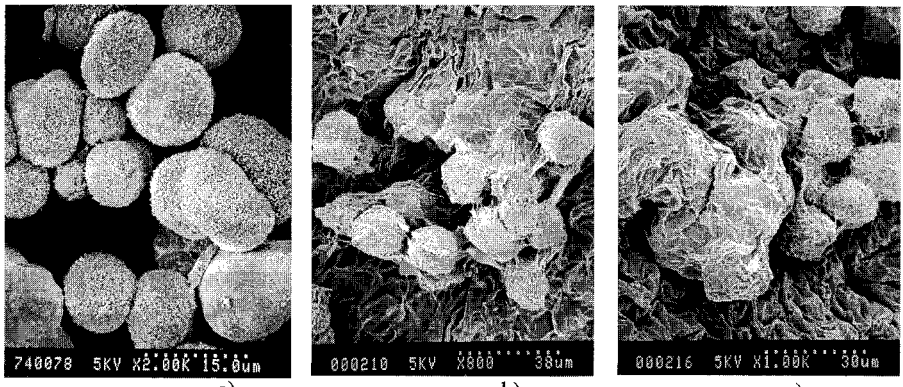


Figure 3: Phase contrast light micrograph (after fixation in osmium tetroxide) of rat hepatocytes encapsulated during free radical polymerization of bifunctional PEO macromonomers at 37°C after 3 hours in culture (magnification X50).



a) b) c)

Figure 4: Scanning electron microscopy micrograph of freshly isolated rat hepatocytes (a), of rat hepatocytes encapsulated during free radical polymerization of bifunctional PEO macromonomers at 37°C after 3 hours (b) and 24 hours (c) in culture.

Figure 4 depicts the rat hepatocytes encapsulated in PEO hydrogel (11500 g.mol-1) after 3 hours in culture (4b) and after 24 hours in culture (4c). Figure 4a shows the freshly isolated rat hepatocytes and serves as control. After 3 hours in culture the cells appeared shrinked and in some cases presented holes in their membrane. This phenomenon was increased after 24 hours in culture. Almost identical conclusions could be drawn for hydrogels prepared from PEO macromonomer precursors of molar mass 15000g.mol^{-1}.

Conclusion

In the present work, PEO macromonomer based hydrogels were tested with respect to their potentiality to serve as a template for the growth of hepatocytes on the surfaces or in the hydrogels. The efficiency of the growth on surfaces is directly related to the physico-chemical characteristics of the hydrogels. Hepatocytes could also be incorporated directly in hydrogels during the crosslinking process. Survival of cells during few hours was observed. For longer periods, the survival shows some problems. Further work is going on along that line. In addition, the possibility to homopolymerize bifunctional PEO macromonomers to hydrogels directly in physiological medium opens new perspectives for the homogeneous incorporation of drugs or cells into biocompatible water swollen matrices.

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