JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 71–75

Evaluation of poly(vinyl alcohol) hydrogels as a component of hybrid artificial tissues

M. G. CASCONE
Dipartimento di Ingegneria Chimica, Università di Pisa, Via Diotisalvi 2, 56126 Pisa, Italy

M. LAUS
Dipartimento di Chimica Industriale e dei Materiali, Università di Bologna, Viale del Risorgimento 4, 40136 Bologna, Italy

D. RICCI
Dipartimento di Biofisica e Ingegneria Elettronica, Università di Genova, Via Opera Pia 11A, 16145 Genova, Italy

R. SBARBATI DEL GUERRA
C.N.R. Istituto di Fisiologia Clinica, Via Savi 8, 56126 Pisa, Italy and C.N.R. Centro Studi Processi Ionici, Via Diotisalvi 2, 56126 Pisa, Italy

Hydrogels are three-dimensional polymeric networks very similar to biological tissues. Many synthetic polymers can be used in preparing hydrogels. Among them poly(vinyl alcohol) (PVA), physically crosslinked by repeated freeze–thawing cycles of polymer aqueous solutions, is widely employed to make hydrogels for biomedical applications. To increase the similarity between hydrogels and natural tissues and to obtain "polymeric hybrid tissues", we attempted to incorporate 3T3 cells, from a mouse fibroblast cell line, into PVA hydrogels obtained by one freeze–thawing cycle using as a solvent complete culture medium. Hydrogels were also made using eight freeze–thawing cycles from PVA solutions prepared using as a solvent either complete culture medium or water. Cell adhesion experiments were performed by seeding 3T3 and human umbilical vein endothelial cells (HUVEC) on to the hydrogel surface. The effect of the solvent and of the different number of freeze–thawing cycles on the mechanical characteristics of the PVA hydrogels were investigated by dynamic-mechanical techniques. A scanning force microscope analysis of the hydrogel surface viscoelastic properties was also carried out. Our results show that PVA is not cytotoxic. Although PVA hydrogel surface characteristics do not seem to favour the adhesion of substrate-dependent cells, encouraging results were obtained with the 3T3 cells incorporation. DMA analysis indicates that the networks prepared by eight freeze–thawing cycles possess a mechanical consistency comparable, even slightly better, than the ones prepared by only one freeze–thawing cycle and used for the cell incorporation studies.

1. Introduction

Hydrogels are three-dimensional polymeric networks able to imbibe large quantities of water or organic liquids without dissolution. The large content of solvent causes hydrogels to have very low interfacial tension with biological fluids. This important feature together with a high permeability to small molecules such as tissue metabolites, and a viscoelastic behaviour, makes hydrogels very similar to biological tissues. For this reason, they have been used as biomaterials in a wide variety of biomedical applications such as ophthalmology, drug delivery, and orthopaedics. Many synthetic polymers can be used in preparing hydrogels. Among them poly(vinyl alcohol) (PVA) is widely employed to make hydrogels for biomedical applications, using a physical crosslinking method consisting of repeated freeze–thawing cycles of polymer aqueous solutions [1]. This freeze–thawing technique offers several advantages over the usual chemical crosslinking or radiation-induced crosslinking. In fact these techniques could compromise the biocompatibility of the materials.

The aim of our study was to increase the similarity between hydrogels and natural tissues to obtain a "hybrid synthetic tissue" with a potential high degree of biocompatibility. Accordingly we have attempted to incorporate 3T3 cells, from a mouse fibroblast cell line, into PVA hydrogels. To reach this goal PVA hydrogels were prepared from 5% PVA solutions using a single freeze–thawing cycle and using complete cell culture medium (CM) as a solvent. 3T3 and human umbilical vein endothelial cells (HUVEC) were also seeded on to

0957–4530

the hydrogel surface to evaluate cell adhesion and growth. The mechanical characteristics of PVA hydrogels were studied by dynamic-mechanical methods and their viscoelastic surface properties by scanning force microscope analysis (SFM).

2. Materials and methods

2.1. Cytotoxicity tests

PVA with molecular weight (MW) of 115 000 and PVA with MW of 72 000, were subjected to cytotoxicity tests. Modified FRAME Protocols [2] were used. Two sets of samples of 100 mg each were prepared: one set was supplemented with 5 ml PBS and then sterilized by autoclave (121 °C). The second set was first sterilized by ethylene oxide, then supplemented with 5 ml PBS and left at 37 °C for 5 days. Extracts were filtered with 0.2 μm cellulose acetate filters and tested for cytotoxicity on 3T3 mouse fibroblast cells, using three different colorimetric methods: neutral red uptake (NRU) assay, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay and the kenacid blue R-binding (KB) method [3].

2.2. Preparation of PVA hydrogels for cellular adhesion and proliferation tests

A solution of 10% PVA (MW = 115 000) was prepared dissolving PVA in PBS solution in an autoclave for 1 h at 120 °C. To this solution was added a blend of CM and dimethylsulfoxid (DMSO) (ratio 4:1) to obtain a final concentration of 5% PVA. This solution was dispensed in two six-well plates (2 ml/well). One plate underwent one cycle of freeze–thawing, consisting of 15 h at − 80 °C and 4 h at 4 °C, the other plate was subjected to eight freeze–thawing cycles. These cycles, with the exception of the first one, consisted of 1 h at − 20 °C and 30 min at 4 °C. The first cycle differed from the others due to a longer standing time at − 20 °C (overnight).

In a series of experiments the hydrogels were coated with either fibronectine or gelatine (50 μl/cm^2) before cell seeding. In other experiments denatured collagen molecules were covalently immobilized on the hydrogel surfaces [4] to favour cell attachment.

The hydrogels were first sterilized by means of a HCl solution, then seeded with either HUVEC or 3T3 cells. HUVEC were previously isolated from the human umbilical vein by trypsin/EDTA treatment and cultured in CM supplemented with 20% human serum following a standard protocol [5]. Seeding density was 4×10^4 cells per cm^2 for the cell adhesion tests and 2×10^4 cells per cm^2 for the cell proliferation tests. The hydrogels were incubated at 37 °C in air with a 5% CO_2 and at 24 and 48 h after seeding were processed for scanning electron microscopy (SEM) analysis.

2.3. Preparation of PVA hydrogels for cellular incorporation tests

A solution of 10% PVA (MW = 115 000) was prepared by dissolving PVA in PBS in an autoclave for 1 h at 120 °C. First, gelatin was added to CM (2% final concentration) and then 3T3 cells. This cellular suspension was then gently mixed with DMSO (ratio 4:1) and with the 10% PVA solution, obtaining a 5% PVA solution containing 3×10^5 cells per ml. This solution was dispensed in a six well plate (1.5 ml/well). The plate underwent a single freeze–thawing cycle consisting of 15 h at − 80 °C and 4 h at 4 °C, to obtain hydrogels. These hydrogels were supplemented with CM and incubated at 37 °C in a 5% CO_2 atmosphere. After 2 and 96 h incubation, the samples were processed for the histological analysis.

2.4. Histological analysis

The cell-containing hydrogels were incubated for 3 h in neutral red (NR) medium, washed, fixed in neutral formalin, embedded in paraffin, stained with hematoxylin-eosin and cut into sections (thickness = 4 μm).

2.5. Preparation of PVA hydrogels for dynamic-mechanical tests and SFM analysis

Two solutions of 10% PVA (MW = 115 000) were prepared dissolving PVA respectively in PBS and water in an autoclave for 1 h at 120 °C. To the first solution was added an equal volume of CM and DMSO (ratio 4:1) to obtain a final concentration of 5% PVA. To the second solution was added an equal volume of water and DMSO (ratio 4:1) to obtain a final concentration of 5% PVA. Each solution was dispensed in two six-well plates (2 ml/well). Two plates underwent one cycle of freeze–thawing, consisting of 15 h at − 80 °C and 4 h at 4 °C. The other two plates underwent eight freeze–thawing cycles. We indicate as CM1 and H_2O1, respectively, the hydrogels made by one freeze-thawing cycle with CM and with water, and as CM8 and H_2O8, respectively, the hydrogels made by eight freeze-thawing cycles with CM and with water.

2.6. Dynamic-mechanical tests

Hydrogels were analysed with a dynamic-mechanical analyser (Perkin Elmer DMA-7), employing parallel plate geometry. Stress scans in the 10–1200 mN range were performed using a static-to-dynamic stress ratio of 150%, at 1 Hz frequency and at a rate of 50 mN/min.

2.7. SFM analysis

The SFM used was a Park Scientific Instrument (PSI) Universal SPM (Sunnyvale, Calif.), equipped with 75 μm and 2.5 μm scanners. For z-axis calibration, we used a home-made tilted silicon plane, with a slope of 10.5° measured by a laser-beam reflection method. All experiments were performed with the sample inserted in the standard liquid cell filled with PBS solution at room temperature and by using the 200 μm long, 0.6 μm thick V-shaped cantilevers supplied by PSI, which had a nominal spring constant $K = 0.032$ N/m. Every set of measurements has been done with a new

tip. The force-versus-indentation curves were calculated by using the return force-versus-distance curves and the method described by Weisenhorn [6]. The force scale for these curves was calibrated by using, as a reference substrate, a piece of silicon wafer inserted next to the sample in the same liquid cell.

3. Results

3.1. Cytotoxicity tests

Cytotoxity experiments showed that the type of sterilization (autoclave or ethylene oxide) did not significantly (one-factor Anova, $p > 0.05$) affect the results. Both PVA with MW of 115 000 and PVA with MW of 72 000 gave results not significantly different (paired t-test $p > 0.05$) from the negative controls, suggesting that both polymers do not induce either lysosomal, mithocondrial or proliferation cell damage.

3.2. Cellular adhesion and proliferation tests

The hydrogels were analysed by means of SEM, 24 and 48 h after seeding. The results of this analysis showed that irrespective of the coating with fibronectin or gelatin, cellular adhesion and growth on hydrogel surfaces was very poor. A higher cellular adhesion was observed on hydrogels in which denatured collagen was covalently immobilized on to the surface (Fig. 1).

3.3. Cellular incorporation tests

Hydrogels prepared from a 5% PVA solution in culture medium with cells, using a single freeze–thawing cycle were analysed by optical microscopy and after histological staining with haematoxylin eosin. Viable cells were present at the inside of the hydrogels, as suggested by their morphology after both 5 and 99 h in culture. At 5 h clear signs of NR incorporation by the cellular lysosomes were detected and mitotic figures were present (Fig. 2a). After 99 h the nuclei were less evident, and signs of lack of membrane integrity were sometimes detected together with a decreased incorporation of NR vital dye (Fig. 2b). From the hystological sections the hybrid materials appeared to be "developing loose connective tissue" in which the cells appeared separated by an amorphous type of intercellular substance (Fig. 2a, b).

Figure 1 SEM micrograph of a patch of 3T3 cells, 24 h after seeding onto one-cycle hydrogel. The cells appear not completely distended. (Original magnification: × 1500).

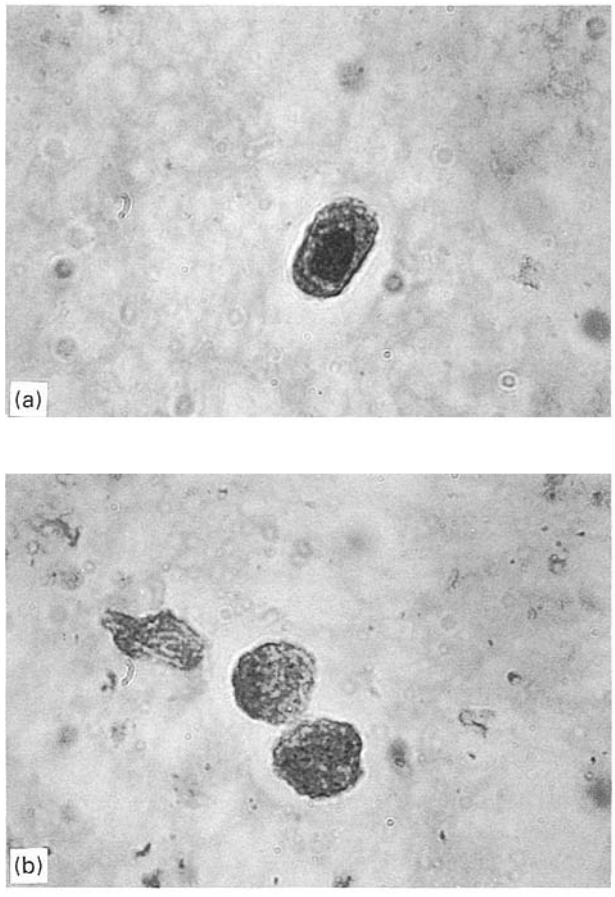

Figure 2 Histological preparations of 3T3 cells incorporated into CM1 hydrogels 24 (a) and 118 (b) hours after incorporation. In (a) the elongated nucleus is typical of a mitotic cell. (Original magnification: × 1000).

3.4. Dynamic-mechanical tests

The trends of storage modulus G', loss modulus G'' and complex viscosity as a function of the dynamic force for the H_2O1 PVA hydrogels are illustrated in Fig. 3a. Both G' and G'' increase, with increasing dynamic force, steeply at first and then more gradually. For no dynamic force value these quantities are independent of the dynamic force. In the whole range of dynamic force investigated a solid-like behaviour (storage modulus, $G' >$ loss modulus G'') is observed. The trend of the complex viscosity closely parallels storage and loss modulus trends. The complex viscosity increases with the dynamic force, approaching a constant value at the highest forces.

The trend in storage modulus G' as a function of the dynamic force for all the poly(vinyl alcohol) hydrogels is illustrated in Fig. 3b. The G' curves of all samples have a shape very similar to the G' curve of the sample previously described. Comparing the various curves, it appears that the samples prepared in the culture medium have a modulus lower than that of those prepared in water. In addition the networks produced with eight freeze-thawing cycles display modulus values higher than the ones prepared with one cycle. To give a more quantitative evaluation of these effects, we

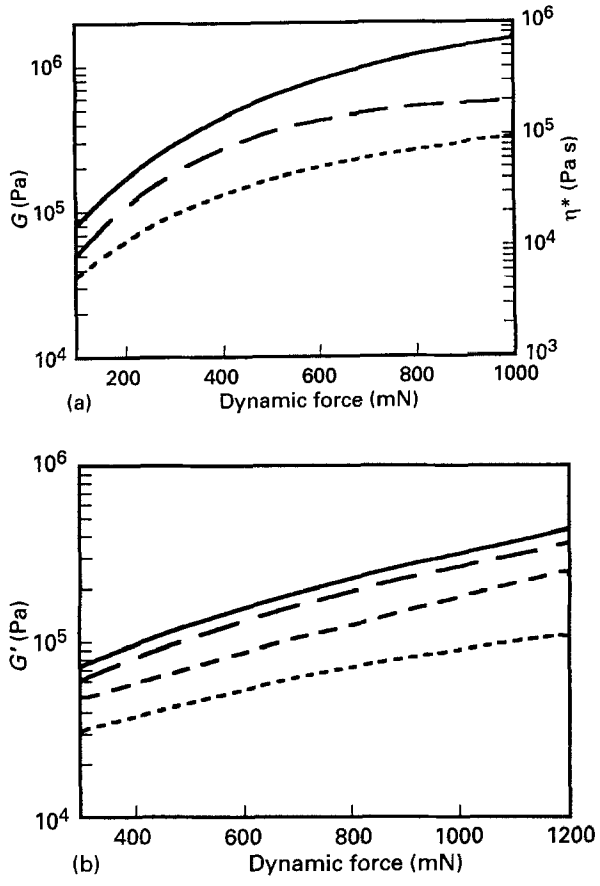

Figure 3 Mechanical characteristics of the PVA hydrogels. (a) The trend of storage modulus G' (——), loss modulus G'' (– –) and complex viscosity (.....) as a function of the dynamic force for the H_2O1 PVA hydrogels. (b) The trend of storage modulus G' as a function of the dynamic force for all the hydrogel types. H_2O8 (——), H_2O1 (– – –), CM8 (– –), CM1 (.....).

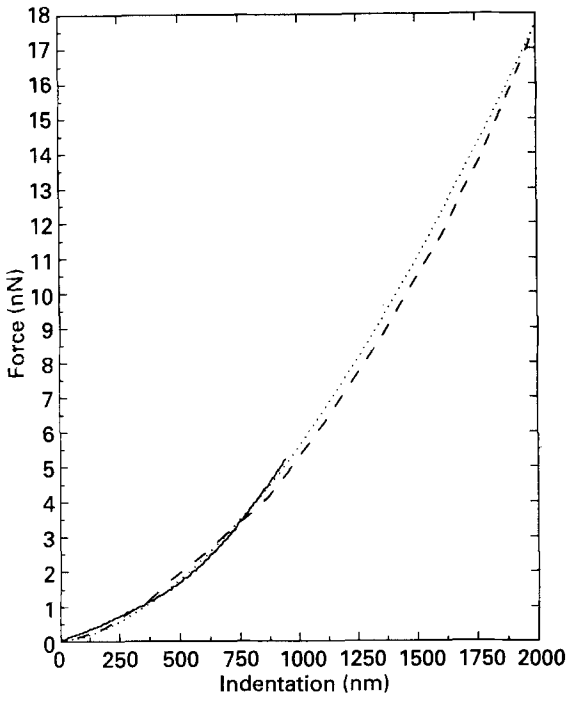

Figure 4 SFM analysis of CM1 hydrogels. The force-versus-indentation curves are reported, corresponding to the 75 μm (——) and the 2.5 μm (– – –) scanners. Each curve is the average of five consecutive measurements on the same spot. (A best fit is shown (.....).

arbitrarily chose a dynamic force of 800 mN and determined the corresponding G' values, marked (G'800) in different samples. The G' (800) values are 7.3×10^4, 1.3×10^5, 1.9×10^5 and 2.3×10^5 for samples H_2O8, H_2O1, CM1 and CM8, respectively.

3.5. SFM analysis

Two series of experiments have been performed using different samples of CM1 hydrogels and the two scanners available on our SFM. Results are reported in Fig. 4 where curve a corresponds to the experiments performed using the 75 μm scanner and curve b to those using the 2.5 μm scanner. No appreciable differences have been observed in other measurements performed at different positions on the sample surface.

The classical elasticity theory of rotationally symmetric, infinitely hard punches [7] gives the following force (F) versus indentation (z) equation for the case of a parabolic-shaped tip with a radius of curvature R at the apex:

$$F_{\text{parabola}} = \frac{4\sqrt{R}}{3(1 - \mu^2)} E z^{1,5}$$

where E is the relative Young's modulus and μ is the Poisson's ratio. Fitting the experimental curves reported in Fig. 4 with the equation $F = az^b$ one obtains $a = 6.2 \times 10^{-5}$ and $b = 1.66$ (in units of nm, nN and GPa). The exponent b hints at a parabolid-shaped tip and allows us to use the previous equation for an evaluation of E. If we suppose a radius of curvature $R = 50$ nm [6] we can estimate $E = 0.005$ MPa.

4. Discussion

Cells in culture incorporated into or grown on to synthetic polymers can lead to the formation of "hybrid artificial tissues". These hybrid tissues, because of the presence of the cells, would be able, after implantation, to create a biologically active interface with the host tissues. One of the major problems concerns the method of anchoring the cells to the material, maintaining their viability without changing their functions.

Our approach to the preparation of "hybrid artificial tissues" involves PVA hydrogels as a synthetic network matrix in which both human (HUVEC) and murine (3T3) cells were seeded on the surface and (3T3) incorporated. Although PVA is not cytotoxic, PVA hydrogel surface characteristics did not seem to particularly favour the interaction with substrate-dependent cells, as those used in our seeding tests.

Our attempt to incorporate the cells into PVA solutions by means of an overnight freeze–thawing cycle at – 80 °C gave two important results. First, hydrogels could be formed with a single freeze cycle of 15 h, not only with the commonly used procedure of eight freeze–thawing cycles. Freeze periods shorter or longer than 15 h were not suitable for hydrogel formation. Second, the fibroblast cells survived this freezing procedure and showed the morphology of viable cells

capable of incorporating NR vital dye for many hours after incorporation. This procedure seems therefore encouraging as a starting point for future attempts aiming at the composition of a hybrid material very similar to a loose connective tissue. Further experiments will have to be performed using cellular metabolic markers to more accurately monitor cell viability over time. A possible limiting event may be the demonstrated poor suitability of PVA for cell attachment.

Although the biocompatibility and permeability of PVA hydrogels are excellent, few studies on the interaction between hydrogel surfaces and cells have been reported [7]. The extent of cellular adhesion is considerably affected by the physical and chemical properties of the substrate surfaces. Proteins such as fibronectin adhere to the substrate surface and could create an integral connection with the receptors on the cellular surface [7]. On the other hand, the hydrogel water content reduces the polymer parenchyma (namely the solid polymer part of the hydrogel), and makes the protein–substrate interaction less effective. For 3T3 cells and other cells that have receptors for proteins like fibronectin, the amount of adsorption of these proteins to the substrate appears to be a critical factor controlling cell interactions with the substrate [8]. In addition it is necessary for a substrate to have a certain rigidity to support the stress caused by the deformation and flattening of the cells [7]. Another critical factor controlling cell adhesion on hydrogels is the porosity. We tentatively suggest that one reason for the poor cell adhesion and growth on our samples is the microporous structure of the hydrogels [9].

To better characterize the PVA hydrogels used for our incorporation and adhesion studies, from a mechanical point of view, we have started a study of the macro- and micro-mechanical features using a conventional DMA and a SFM. The DMA analysis indicates that the overall network consistence is strongly dependent on both the preparative thermal treatment and the solvent. In particular, the network prepared in water showed a mechanical consistency higher than the one prepared in culture medium. In addition the eight- cycle procedure afforded more stable networks than those prepared with one cycle only (Fig. 3a, b). Nevertheless, it seems from our cell adhesion tests that the mechanical consistency of all the investigated samples is inadequate to support cell adhesion and growth.

A preliminary micro-mechanical investigation was performed by scanning force microscope (SFM) (Fig. 4). Measurements of force-versus-indentation allowed calculation of the local Young's modulus according to indentation theory [10]. To evaluate the potential of the technique in providing information on the local mechanical micro-characteristics of hydrogels we have performed a force-versus indentation study of the sample CM1.

The value $E = 0.005$ MPa calculated for our CM1 samples is orders of magnitude smaller than that of rubbers (0.16 MPa) and polyurethane (14 MPa) and is comparable to the Young's modulus of living cells (0.003–0.013 MPa).

In conclusion, the CM1 PVA hydrogels seem to constitute a promising synthetic network matrix that allows cell incorporation and possess mechanical properties not very dissimilar to those of PVA hydrogels prepared in water by the classical eight- cycles procedure.

Acknowledgements

The authors thank A. Bonaretti for technical help, V. Gattai and L. Pozzolini for hystological preparations, P. Narducci for SEM analysis, and professor B. Bianco and Dr W. Pelosi for helpful discussion and suggestions. This work was partially supported by the Italian Ministry for University and Research (MURST).

References

1. M. NAMBU, Eur. Pat. N° 0058497 B1 (1985).
2. INVITTOX, "The Ergatt/Frame data bank of *in vitro* techniques in toxicology" (Inucltox, Nottingham, England).
3. M. G. CASCONE, P. CERRAI, M. TRICOLI and R. SBARBATI DEL GUERRA, *Cytotechnol.* **11(S1)** (1993) 137.
4. Y. TABATA, S. V. LONIKAR, F. HARII and Y. IKADA, *Biomaterials* **7** (1986) 234.
5. R. SBARBATI, D. GIANNESSI, M. C. CENNI, G. LAZZERINI and R. DE CATERINA, *Int. J. Artif. Org.* **14** (1991) 491.
6. A. L. WESENHORN, M. KHORSANDI, S. KASAS, V. GOTZOS and H. J. BUTT, *Nanotechnol.* **4** (1993) 106.
7. S. NAGAOKA, H. TANZAWA and J. SUZUKI, *In Vitro Cell. Dev. Biol.* **25** (1990) 51.
8. T. A. HORBETT and M. B. SCHWAY, *J. Biomed. Mater. Res.* **22** (1988) 763.
9. P. GIUSTI, L. LAZZERI, N. BARBANI, A. BONARETTI, M. PALLA and L. LELLI, *J. Mater. Sci. Mater. Med.* **4** (1993) 538.
10. I. N. SNEDDON, *Int. J. Engng Sci.* **3** (1975) 47.