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Biological characterization of hydrogels of poly(vinyl alcohol) and hyaluronic acid

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Hydrogels of hyaluronic acid (HA) and poly(vinyl alcohol) (PVA) were prepared using eight freezing–thawing cycles from HA/PVA blends (10/90, 20/80, 30/70, 40/60, 50/50, and 0/100 (w/w) ratios). The biocompatibility of the hydrogels was tested by means of *in vitro* cytotoxicity and cytocompatibility tests using cell culture techniques. The release with time of HA and PVA, the two hydrogel components, ion aqueous medium was also monitored and evaluated. The results indicate that all the hydrogels are not cytotoxic, while cell adhesion was very scarce in PVA and was not improved by the addition of HA. The release kinetics of HA and PVA from the hydrogels were different. After 2 h, HA percentages from about 80 (10/90) blend) to 100% (20/80, 40/60 blends) were released from the hydrogels into the aqueous medium. In contrast, the percentages of released PVA remain lower in time compared with HA, reaching a plateau after 24 h and ranging from a maximum of about 13% (0/100 blend) to a minimum of about 6% (10/90, and 20/80 blends).

1. Introduction

Hydrogels form a class of materials that are potentially useful for the replacement of soft tissues. They consist of three-dimensional polymeric networks held together by cross-links of covalent bonds and weak cohesive forces, especially in the form of hydrogen bonds. These networks are able to absorb large quantities of water or organic liquids without disintegration. The large content of solvent causes hydrogels to have very low interfacial tension with biologic fluids. Among the many synthetic polymers that can be used to prepare hydrogels, poly(vinyl alcohol) (PVA) is one of the most widely employed [1] in the field of biomedical applications. Various cross-linking methods can be employed to obtain PVA hydrogels. Our attention was focused on the method of Nambu [2]. This is a physical method of cross-linking consisting of repeated freezing and thawing cycles of aqueous solutions of the polymer that does not require any additional chemicals or cross-linking agents. According to some of the most recent results obtained in our laboratory [3], improvements in the characteristics of synthetic biomaterials could be achieved by the addition of biological macromolecules. For this purpose we believe that biopolymers contained in the extra cellular matrix, such as hyaluronic acid (HA), could be usefully employed. HA is a polysaccharide that influences several cellular functions such as migration, adhesion, and proliferation. We prepared PVA hydrogels containing various percentages of HA. These "bioartificial materials" [3] were tested for biocompatibility, i.e. cytotoxicity and cytocompatibility. The release with time of the two hydrogel components PVA and HA in an aqueous medium was also monitored and evaluated.

2. Materials and methods

2.1. Hydrogel preparation

A 5% w/v PVA (powered, Aldrich Chemie, with molecular weight of 114000) solution in water was prepared by adding the solid PVA to distilled water into a flask equipped with a reflux condenser, then gradually raising the temperature from room temperature, to the boiling point of the solution, by means of an oil-bath heater at 120 °C. The dissolution of PVA was complete in 2 h. A 5% w/v HA (sodium salt with molecular weight of 250000, FABS SpA Italy) solution in water was prepared by adding the solid powdered HA to distilled water, and raising the temperature to 60 °C. The dissolution of HA was completed in 2 h. HA/PVA blends with 10/90, 20/80, 30/70, 40/60, 50/50, 0/100 (w/w) ratios were prepared. The final PVA content was kept at 2.5%. Each blend was dispensed in a 24-well plate. Samples underwent eight cycles of freezing–thawing to obtain hydrogels. With the exception of the first one, each cycle involved lowering the temperature to − 19 °C, standing at this temperature for 1 h, then raising the temperature to room temperature. The first cycle (the same for all

samples) differed from the others because of a longer standing time at − 19 °C (12 h).

2.2. Cytotoxicity tests

Samples were sterilized by ethylene oxide and to each was added 10 ml phosphate-buffered saline solution (PBS); and they were then kept at 37 °C for 5 days. Extracts were filtered with 0.4 μm cellulose acetate syringe filters (Nalgene, U. S. A.) and tested for cytotoxicity on 3T3 mouse fibroblast cells cultured in Dulbecco's Minimal Essential Medium with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 5 μg/ml fungizone. The cells were seeded onto 96-well plates at two different densities: 1.2×10^4 cells/cm^2 for the 24 and 6×10^3 cells/cm^2 for the 72 h exposure period assays. Three different colorimetric methods were used: 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 [4]. NRU was used to assess cell lysosomal damage, MTT to verify mitochondrial functionality and KB for total protein determination. The extracts were added to the wells (100 μl/well) 24 h after seeding. The plates were then incubated for 24 or 72 h at 37 °C in a CO_2 atmosphere. 2,4-dinitrophenol (70 μg/ml) was added to the positive control wells. After the incubation period, the procedures were different for the three assays. NRU assay: the culture medium was replaced with 150 μl NR medium per well. The plates were incubated for 3 h at 37 °C, the NR medium was removed, the cells were rinsed twice with PBS and 150 μl destain solution (1% glacial acetic acid + 50% ethanol + 49% distilled water) per well were added. The plates were shaken for 10 min and the absorbance was read at 540 nm against a reference well which contained no cells. MTT assay: the culture medium was added with 10 μl MTT solution per well. After 4 h of incubation the solution was removed, 100 μl DMSO per well were added and, after a 5 min slow agitation, the absorbance was read at 550 nm. KB assay: the culture medium was replaced with 150 μl KB dye per well. The dye was aspirated after a 20 min slow agitation and the wells washed twice with a washing solution. The desorbing solution was then added and the plates shaken for 20 min. The absorbance was read at 570 nm.

2.3. Cytocompatibility tests

After sterilization by means of HCl solution, the hydrogel pH was brought again to physiological values. Hydrogels were then seeded with either 3T3 fibroblast cells or human umbilical vein endothelial cells (HUVEC). HUVEC were previously isolated from the umbilical vein by trypsin/EDTA treatment and cultured in a serum-supplemented culture medium following a standard protocol [5]. Seeding density was 8×10^4 cells per cm^2. The hydrogels were incubated at 37 °C in an atmosphere of air and 5% CO_2 for 24 or 48 h and then processed for scanning electron microscopy (SEM) analysis [5].

2.4. Releasing tests

Releasing tests were carried out to evaluate the amounts of both PVA and HA released from the hydrogels in aqueous solution. Each hydrogel was immersed in 30 ml of distilled water, for 98 h at 37 °C. At 2, 4, 8, 24, 48, 76 and 98 h, the releasing solution was removed, used for PVA and HA release measurements and replaced with 30 ml of fresh distilled water. The determination of released PVA was carried out spectrophotometrically according to the method of Bujanda and Rudin [6].

Released HA was estimated by weighing the solid content of the releasing solution after evaporation and subtracting the fraction due to PVA.

3. Results

3.1. Cytotoxicity tests

The results of our tests suggested that no cytotoxic effects (paired t-test, $p > 0.05$) were exerted on the cell lysosomal, mitochondrial or proliferation activity by all the material extracts after 72 h incubation (Fig. 1b). The results after 24 h incubation of the extracts with the 3T3 cells showed more variability (Fig. 1a). In particular in the NRU test the hydrogels 20/80 and 30/70 performed significantly better than their negative controls ($p < 0.01$ and < 0.05, respectively).

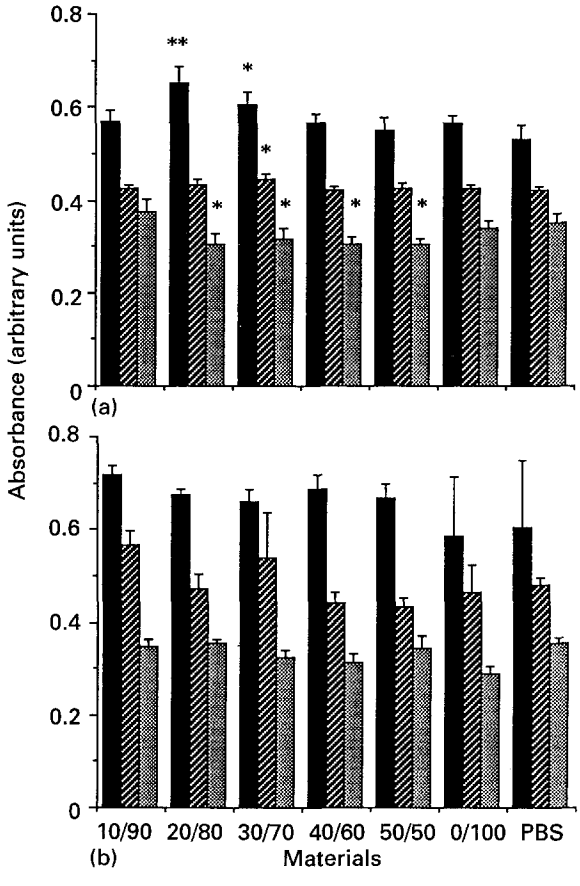

Figure 1 Viability of 3T3 mouse fibroblasts is measured as a function, respectively, of Neutral Red (NR, ■*) uptake into the lysosomes, mitochondrial integrity (MTT,* ▨*) and total protein content (KB,* ▦*) after 24 (a) and 72 h (b) incubation of the cells with hydrogels extracts. The absorbance was read at 540 (NR), 550 (MTT), and 570 nm (KB). Each column represents the mean of five points ± SE. Paired t-test, significant at the *95%, and **99% levels.*

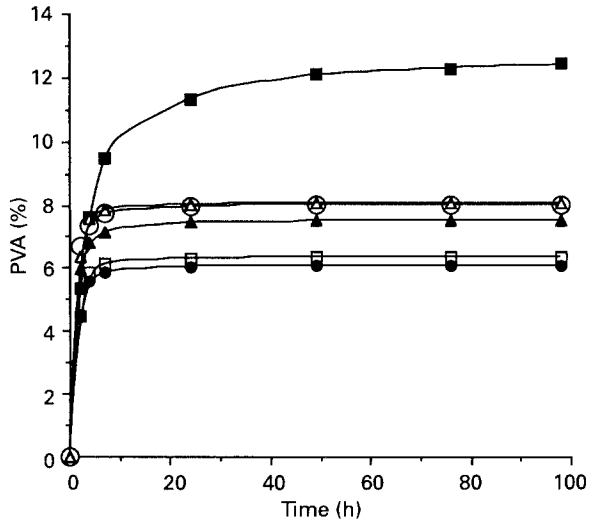

Figure 2 The percentage of released PVA, normalized to the initial PVA content of the hydrogels, reported as a function of time. Each point is the mean of two determinations (HA/PVA blend: ■ 0/100; □ 10/90; ● 20/80; ○ 30/70; ▲ 40/60; △ 50/50).

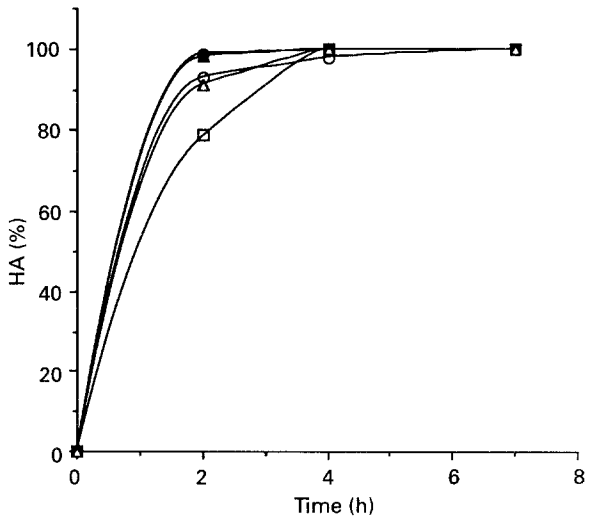

Figure 3 The percentage of released HA, normalized to the initial HA content of the hydrogels, reported as a function of time. Each point is the mean of two determinations (HA/PVA blend: □ 10/90; ● 20/80; ○ 30/70; ▲ 40/60; △ 50/50).

A similar result was achieved by the 30/70 hydrogels in the MTT test ($p < 0.05$). In contrast, in the KB test, hydrogels 20/80, 30/70, 40/60, and 50/50 performed slightly but significantly worse ($p < 0.05$ than their negative controls.

3.2. Cytocompatibility tests

At the defined time intervals after cell seeding, the hydrogels were analysed by means of SEM. The results of this analysis showed that on all the hydrogels, independently of the composition, cellular adhesion was very poor.

3.3. Releasing tests

The percentage of released PVA, normalized to the initial PVA content of the hydrogels, is reported as a function of time in Fig. 2, where each point is the sum of the PVA amounts up to that sampling time. The PVA release reached a plateau after 24 h for all hydrogels, with the exception of the pure PVA hydrogel, which was still slightly releasing after 98 h.

The PVA amounts released from all HA-containing hydrogels ranged from 6 to 8% and were lower than that released from the pure PVA hydrogel (13%). Among them, the lowest values were observed for the 10/90 and 20/80 HA/PVA samples.

Fig. 3 shows the percentages of released HA, normalized to the initial HA content of the hydrogels. It was observed that HA release occurred at a higher rate than PVA, for all the hydrogels. Amounts of HA ranging from 80% to 100% of the initial HA content were released after 2 h.

4. Discussion

The PVA release data show that the quantities of removed PVA in HA-containing hydrogels were lower than those in pure PVA samples (Fig. 2). Almost the total HA content in the hydrogels was released after 7 h (Fig. 3), while the amount of released PVA was much more limited in percentage than the HA and reached a plateau at 24 h. This different behaviour might lead to the hypothesis that during hydrogel formation by freezing HA chains act as crystallization sites for PVA chains, without formation of HA/PVA bonding. This effect seemed more marked in 10/90 and 20/80 samples. Small percentages of HA could be capable of producing the best PVA crystallite formation [7]. HA percentages higher that 20% still produce the effect of favouring crystallization but to a lower extent [7]. This is probably because HA content higher than 20% exerts a perturbing action in the formation of PVA crystallites.

Both PVA and HA components are non-cytotoxic (Fig. 1). When present in combination, only after 24 h incubation a certain variability of results (compared with the negative control) was present within the different tests. This finding is often present at 24 h incubation and was previously attributed by us to a metabolic instability of the cells. Therefore the results after 72 h incubation are those that must especially be taken into account. Cytocompatibility, i.e. the capability of supporting cell adhesion and growth was poor. This agrees with previous observations on PVA hydrogels [8]. These results seem to point to the direction of a possible utilization of HA/PVA hydrogels as drug releasing systems. The capability of PVA and HA to be released according to modalities different in time and amount could be coupled to the necessity of controlled differential drug release. Recently it has been reported that in a similar system the controlled release of growth hormone was directly proportional to the HA content [9].

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