Biocompatibility of poly(vinyl alcohol)-hyaluronic acid and poly(vinyl alcohol)-gellan membranes crosslinked by glutaraldehyde vapors

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Glutaraldehyde (GTA) solutions are commonly used to crosslink biomolecules and artificial polymers in order to reduce the degradation rate and to avoid the rapid dissolution in biological fluids. The toxicity of these materials is often due to the presence of GTA residuals unremoved by washing procedures. In this study membranes of PVA-hyaluronic acid and PVA-gellan with different composition have been obtained by solution casting technique and crosslinked by exposure to vapors of GTA in acid environment. The harmful effects of GTA residuals released from the membranes have been evaluated by the cytotoxicity and cytocompatibility *in vitro* tests, based on the cell culture method.

The results showed that these materials have no toxic effects: they do not affect cell viability and proliferation, nor exert damages on mithocondrial and lysosomal functions. The poor adhesion of cells seeded directly onto membranes is due to the surface properties of these materials which are completely refractory at cell adhesion and proliferation.

The use of GTA in vapor phase as crosslinking agent of natural and artificial polymer blends is demonstrated to be an efficacious procedure that avoids the presence of toxic residuals into materials.

1. Introduction

In recent years many researchers have focused their attention on materials called ''bioartificial polymeric materials'' [1]. These new processable materials based on blends of biological and synthetic polymers join together good biocompatibility characteristics with good physical and mechanical properties.

Hyaluronic acid [2] and gellan [3, 4] have been used in association with artificial polymers, such as poly(vinyl alcohol) (PVA) or poly(acrylic acid) (PAA) in order to obtain films and hydrogels [5–8].

These blends must to be crosslinked in order to reduce the degradation rate of biomolecules and artificial polymers and to avoid the rapid dissolution in biological fluids.

Although highly toxic, the use of aldehydes as crosslinking agents is very frequent. Glutaraldehyde (GTA) solutions, for example, are commonly used to crosslink collagen-based biomaterials used for the treatment of burn wounds [9–11] in order to modulate the resorption time of collagen, and to crosslink polymers such as poly(vinyl alcohol) [12].

A large variety of reaction pathways are involved in the reaction mechanism of crosslinking [13]. GTA is still the most widely used fixative for bioprosthetic heart valves, but at the same time, it is also believed to be partly responsible for tissue calcification and lack of surface reendothelialization. Current studies are addressed to individuate detoxification treatments of aortic wall tissue [14] or to found alternative crosslinking techniques [15] or natural crosslinking reagents [16].

The toxicity is due to the presence in the materials of residuals of GTA unremoved by washing procedures.

In this study membranes of PVA-hyaluronic acid and PVA-gellan with different composition have been obtained by solution casting technique and crosslinked by exposure to vapors of GTA in acid environment. The harmful effects of this treatment has been evaluated by the cytotoxicity and cytocompatibility in vitro tests, based on the cell culture method.

The cytotoxicity tests, that evaluate the viability and metabolic activity of 3T3 fibroblasts cultured with material extracts (indirect tests), have been performed using three different colorimetric methods: Neutral Red uptake assay (NR) to assess cell lysosomal damage, MTT assay to verify mitochondrial functionality and Kenacid Blue R-Binding method (KB) for total protein content determination. The rationale of these tests is that cells in culture proliferate at a known optimal rate, which would be reduced by chemical residuals affecting one or more essential functions, such as mitochondrial activity, DNA synthesis, maintenance of the membrane integrity or protein synthesis [17].

In the cytocompatibility test the cells are seeded

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directly onto the material surfaces in order to study the cell adhesion and proliferation (direct test).

2. Materials and methods

2.1. Materials and membrane preparation

Hyaluronic acid (HA, Mw 180.00–200.000) was supplied by FAB (Abano Terme, Italy), gellan by Sigma (Italy) and poly(vinyl alchol) (PVA, Mw 85.000–146.000, hydrolysis degree 99%) by Aldrich (Germany).

PVA and HA aqueous solution at 2% (w/v) were mixed to obtain blends of PVA-HA with different compositions (100/0, 80/20, 50/50).

PVA and gellan aqueous solution at 1% (w/v) were prepared at 90 °C and mixed at the same temperature in five different ratio (100/0, 70/30, 50/50, 30/70, 0/100).

Membranes of PVA-HA and PVA-gellan were obtained by solution casting at room temperature and crosslinked by exposure to glutaraldehyde (8%, w/v) and HCI (6%, w/v) vapors for 32 h at 37 °C.

The membranes were rinsed for 30 min in phosphate buffer saline (PBS) in sterile condition to eliminate the residual glutaraldehyde.

2.2. SEM analysis

In order to evaluate the membrane surface morphology, the samples were sputter-coated with gold and examined by a scanning electron microscope (Philips XL 40) at 5 KV acceleration voltage.

2.3. Cell cultures

Mouse fibroblast 3T3 cell line (ATCC, USA) were cultured in Dulbecco Modified Eagle's Medium (DMEM, Biowhittaker, Belgium), containing penicillin/streptomycin (100/100 U), amphotericin B (2.5 μg/ml) and gentamycin (100 μg/ml), supplemented with 10% foetal calf serum (Mascia Brunelli, Italy), and kept at 37 °C in an atmosphere of 5% CO_2 and 99% humidity. Media were changed every three days.

2.4. Cytotoxicity studies

The viability and metabolic activity of cells, previously incubated with material extracts, was checked by the Neutral Red (NR) uptake assay, the Kenacid Blue R (KB) binding method, and the 3-(4,5 dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (indirect tests).

NR is taken up into the lysosomes by viable cells, MTT into the mitocondria. The KB binding assay measures the total cell protein content of the test wells.

Extracts were obtained from materials under standardized conditions (ISO 10993-5). The materials were dipped in borosilicated glass tubes containing a complete culture medium for 120 h, at 37 °C without shaking. The ratio between the sample surface and the volume of the extraction vehicle was 2 cm^2/ml. A glass tube containing the same extraction vehicle with no material was processed according to the same conditions and the corresponding extract provided the negative control for the testing procedure. A toxic control was performed on the same negative control but containing 0.1% phenol solution. The pure extracts (100%), 50% and 20% diluted, were added at cells, seeded 24 h before in 96 multiwell plates. After three days of incubation, viability and metabolic activity of cells were tested by the MTT, NR and KB assays respectively.

2.4.1. MTT assay

The medium was replaced with 50 μl/well of MTT solution (1 mg/ml in culture medium without phenol red). After 4 h of incubation at 37 °C the solution was removed, 100 μl/well of DMSO were added and after 10 min of slow shaking the absorbance was read at 540 nm [18].

2.4.2. NR assay

The medium was replaced with 100 μl/well of NR solution (0.01% in culture medium) and the plates were incubated for 3 h at 37 °C. Then the cells were rinsed with PBS and 100 μl/well of destain solution (1% glacial acetic acid, 50% ethanol, 49% distilled water) were added. The plates were shaken for 10 min and the absorbance was read at 540 nm.

2.4.3. KB assay

The cells were fixed with glutaraldehyde (2.5% in PBS) and then stained with KB dye for 20 min. Then the wells were rinsed twice with a washing solution. The desorbing solution was added and the plates shaken for 20 min. The absorbance was read at 570 nm [19].

2.5. Cytocompatibility studies

For the direct contact method, the membranes were cut in disk (15 mm in diameter), located in a 24-multiwell plate and fixed to the bottom of the wells by a small amount of an inert, sterile, high temperature vacuum grease (Dow Corning, USA).

After rinse on PBS for 30 min, the samples were seeded with fibroblasts 3T3 at 2.0×10^4 cells/cm^2 cell density. The tissue culture polystyrene was used as control. The samples were incubated at 37 °C in humidified atmosphere containing 5% CO_2. Five days after seeding, cell proliferation was evaluated by MTT assay and the cell morphology was performed by light microscopy.

2.6. Statistical analysis

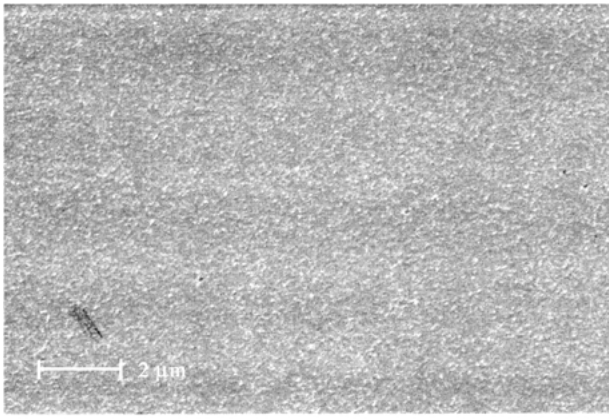
Results were expressed as mean values ± standard deviation for each group of samples. After the assessment of significant differences by one-way variance analysis (ANOVA), differences among groups were established by the Student's *t*-test by a two population comparison. Statistical significance was considered at a probability $p < 0.05$.

3. Results

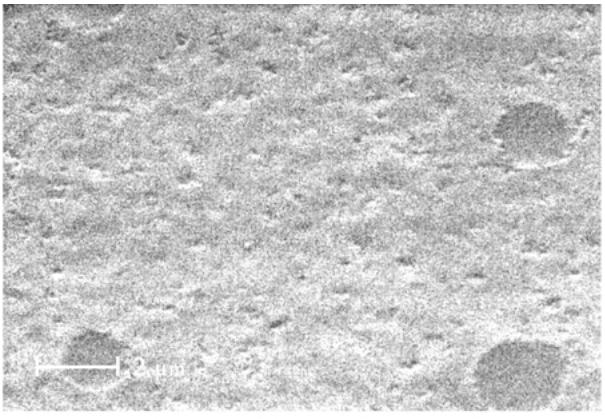
At the SEM investigation PVA/HA membranes appeared dense and the morphology was more irregular with the addition of HA (Fig. 1). On the contrary the PVA-gellan membranes showed an uniform and compact structure independent from the blend composition.

In Figs 2 and 3 are reported the cytotoxicity results concerning the PVA-HA and PVA-gellan membranes respectively. The cell viability after incubation of fibroblasts with three different dilutions of extracts was studied. The MTT, NR and KB assays showed that the cell viability, expressed by the absorbance values, was higher than the positive control containing a phenol solution and similar to the negative control. No differences among the dilutions of extracts was detected, having the undiluted extracts no toxic effects. The statistical analysis confirms this trend.

In Fig. 4 are shown the results regarding the cell proliferation when the fibroblasts were seeded directly onto materials. The cells growth, evaluated by MTT assay, is expressed as percentage with respect to control. The level of absorbance resulted significantly lower with respect to control indicating a poor capability of adhesion and proliferation of the cells on the membranes. The presence of biological polymers, hyaluronic acid and gellan in the blends did not affect the interactions between cells and materials. Fig. 5 shows the cells behavior and their morphology observed by light microscopy. The fibroblasts were located around the membrane of PVA-HA 100/0 whereas they were completely absent onto the material. Moreover no signs of cell damage was monitored. This behavior was observed for all the membranes independently from the presence and amount of the biopolymers in the blends.



(a)



(b)

Figure 1 SEM images of PVA-HA 100/0 (a) and PVA-HA 80/20 (b) membranes (original magnification 8000 ×).

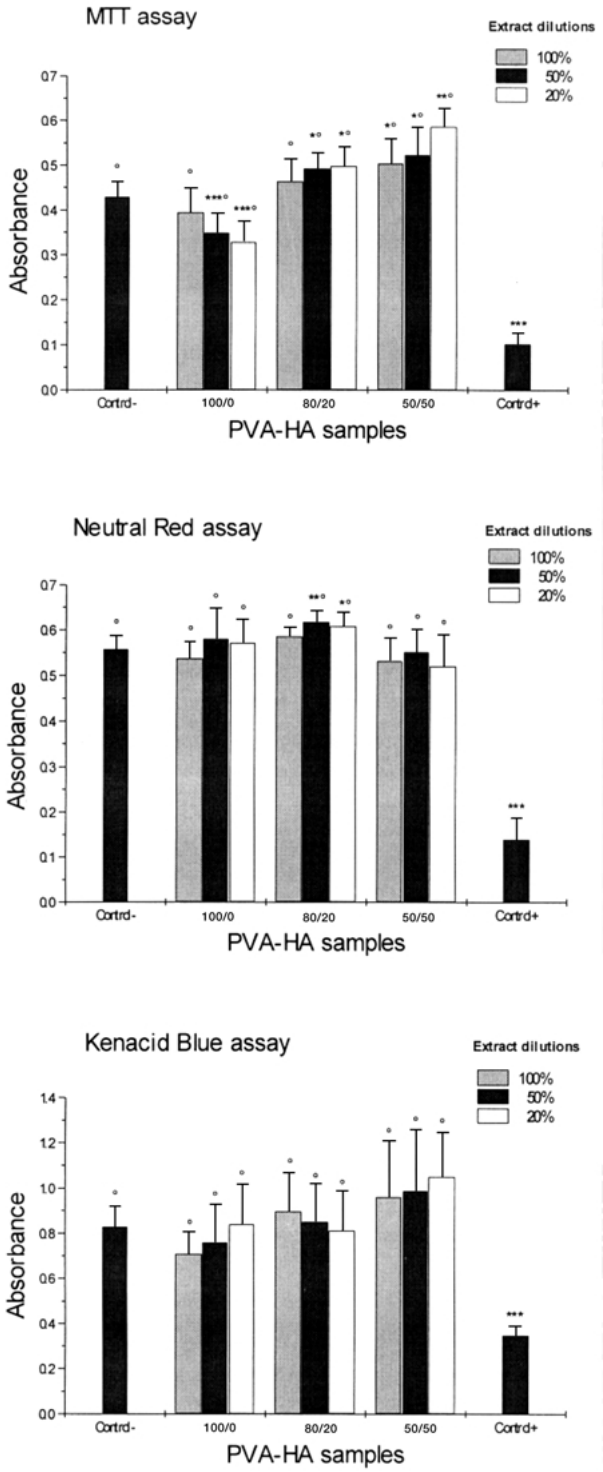
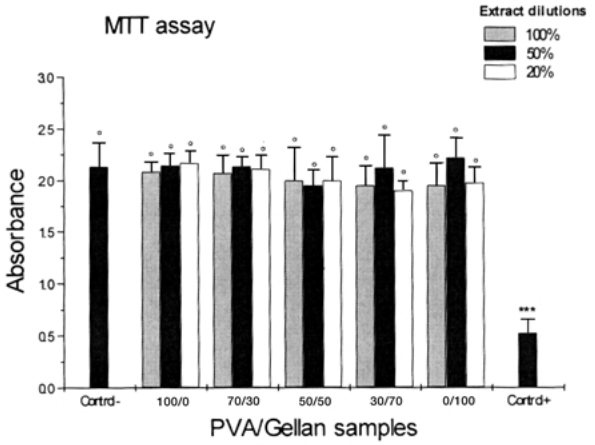
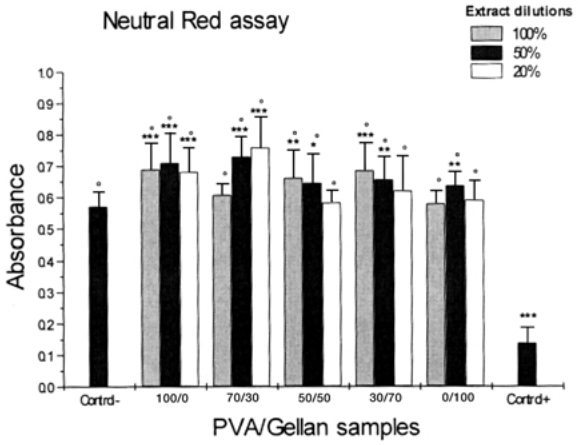


Figure 2 Cytotoxicity evaluation of PVA-HA membranes at different extract dilutions by means of MTT, NR and KB assays. Difference from control − : $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Difference from control + : $^{\circ}p<0.001$.





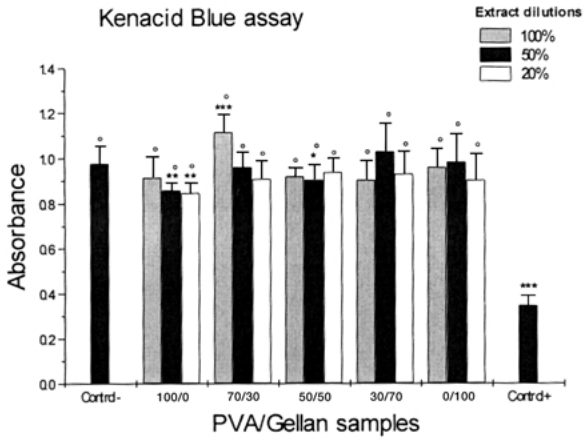
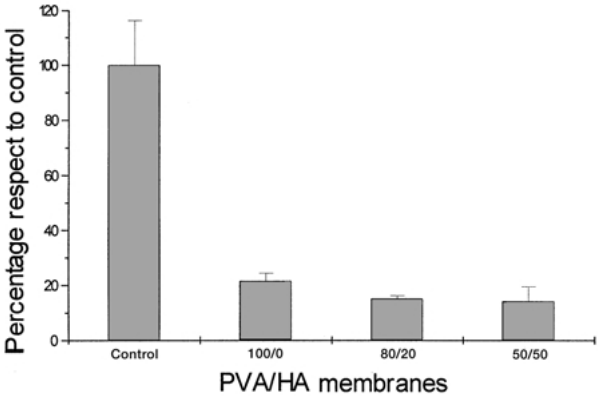


Figure 3 Cytotoxicity evaluation of PVA-gellan membranes at different extract dilutions by means of MTT, NR and KB assays. Difference from control −: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Difference from control +: $^{\circ}p<0.001$.

4. Discussion

The results of the cytotoxicity assays showed that the extracts obtained from the samples have no toxic effects: they do not affect cell viability and proliferation as shown by the total protein content (KB assay), nor exert some damage on mithocondrial and lysosomal function (MTT and NR assay respectively).



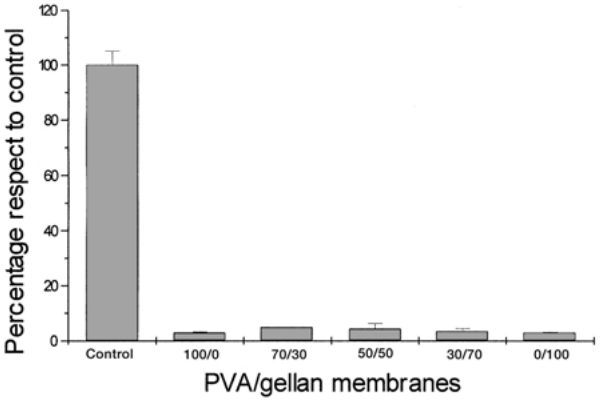


Figure 4 Cytocompatibility test: the growth of fibroblasts onto PVA-HA and PVA-gellan membranes was detected by MTT assay after five days of incubation.

With regard to the PVA-HA 100/0 sample, in MTT assay a small decrease of absorbance with respect to the negative control occurs. This behavior can be neglected because the lowering of cell viability was monitored only for the more diluted extract. On the contrary, an increase of absorbance occurs for some samples.

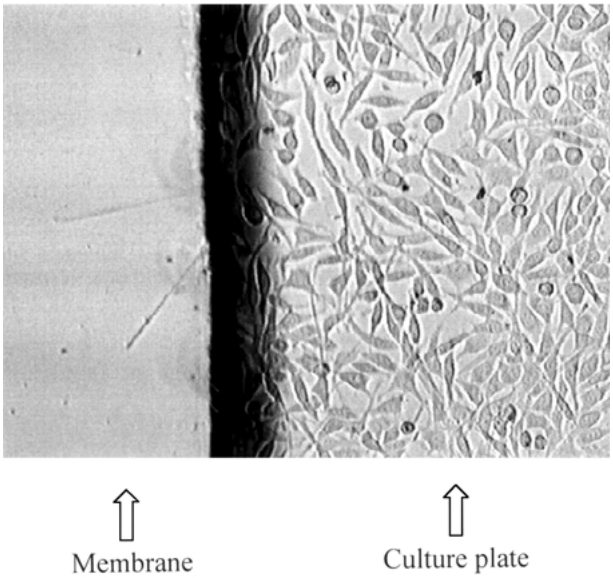


Figure 5 Morphology of fibroblasts cells seeded on PVA-HA 100/0 membrane observed by light microscopy (original magnification 300 ×).

The adhesion of cells seeded directly onto membranes is very poor. As confirmed by the indirect tests, this behavior is not due to the material toxicity, but to the fact that the cells seeded prefer to grow on a culture plate rather than on membranes. Moreover, the cells localized at close contact with the membranes have not signs of damage. In any case the low cell adhesion on surface of these materials has been reported elsewhere [20]. The presence of biological polymers, HA and gellan, in different amount in the blends, does not change the surface properties of membranes which are completely refractory at cell adhesion and proliferation. Membranes morphology appears very little modified from the biopolymer presence; the irregularity and porosity of surface is not enough to promote cell adhesion.

5. Conclusion

In conclusion the use of GTA in vapor phase as crosslinking agent of natural and artificial polymer blends and the following rinse for 30 min in PBS are an efficacious procedure that avoids the release of GTA toxic residuals from materials.

Moreover, the GTA allows to sterilize the membranes avoiding the use of the typical methods of sterilization, such as autoclave, ethylene oxide, gamma radiation, that often damage the polymer structure.

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