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Biocompatible hydrogels based on hyaluronic acid cross-linked with a polyaspartamide derivative as delivery systems for epithelial limbal cells

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ABSTRACT

The aim of this work was to evaluate the potential use of hydrogels based on hyaluronic acid (HA) chemically cross-linked with α,β-poly(N-2-hydroxyethyl) (2-aminoethylcarbamate)-D,L-aspartamide (PHEA-EDA) as substitutes for the amniotic membrane able to release limbal cells for corneal regeneration. Hydrogels, shaped as films, with three different molar ratios (*X*) between PHEA-EDA and HA (*X* = 0.5, 1.0 and 1.5) have been investigated. First, it has been evaluated their swelling ability, hydrolytic resistance in simulated physiological fluid and cell compatibility by using human dermal fibroblasts chosen as a model cell line. Then adhesion studies in comparison with collagen gel, have been performed by using immortalized cells, such as human corneal epithelial cells (HCEC) or primary cells, such as rabbit limbal epithelial cells (RLEC) and/or rabbit limbal fibroblasts (RLF). HA/PHEA-EDA hydrogels allow a moderate/poor adhesion of all investigated cells thus suggesting their potential ability to act as cell delivery systems. Finally, commercial contact lenses have been coated, in their inner surface, with each HA/PHEA-EDA film and it has been found that in these conditions, a greater cell adhesion occurs, particularly when RLEC are in co-culture with RLF. However, this adhesion is only transitory, in fact after three days, viable cells are released in the culture medium thus suggesting a potential application of HA/PHEA-EDA hydrogels, for delivering limbal cells in the treatment of corneal damage.

1. Introduction

Corneal disease is a common cause of blindness in both adults and children. It has been reported that 1.5–2 million cases of monocular blindness worldwide per year are caused by ocular trauma and corneal ulcerations (Whitcher et al., 2001). Moreover the loss of corneal transparency causes poor vision in about 135 million people worldwide. The transparency of the cornea is maintained by the stem cells of the corneal epithelium in the limbal region (Daniels et al., 2006); damage to the limbus may therefore lead to partial or total limbal stem cell deficiency that, in severe cases, may lead to blindness (Dua et al., 2003).

Human amniotic membrane is commonly used as a carrier for delivering cultured limbal stem cells to the cornea essentially after its breaking down on the eye thus leaving limbal cells in place (Arora et al., 2004). It is also used as a cell-free bandage due to its anti-angiogenic and anti-inflammatory properties (Hao et al., 2000).

However, the biodegradation time is not constant as it depends on the processing of the amniotic membrane and on the particular storage regimes used in the tissue banks (Koizumi et al., 2002). Biodegradation also depends very much on the conditions of the patient's eye to which the amniotic membrane is transplanted. Also in spite of extensive screening of the maternal donors before the membrane is used, there is still some risk of viral disease transmission (Pratoomsoot et al., 2008) that cannot be completely eliminated.

Therefore, there is a clinical need to develop a synthetic, biocompatible and slowly biodegradable material which could be used as substitute for the amniotic membrane allowing both the attachment of the limbal stem cells and their subsequent delivery onto

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the cornea. With this aim, contact lenses (Deshpande et al., 2009), recombinant collagen (Dravida et al., 2008), electrospun scaffolds (Deshpande et al., 2010), temperature sensitive polymers (Nishida et al., 2004) and polymer gels (Sudha et al., 2006) have all been investigated. The use of a synthetic material should eliminate the risk of infections and if biodegradable, it is possible to control the degradation rate by varying the fabrication parameters.

For these reasons, the aim of this work was to investigate use of hydrogels, shaped as films, based on hyaluronic acid (HA) chemically cross-linked with α,β-poly(N-2-hydroxyethyl) (2-aminoethylcarbamate)-D,L-aspartamide (PHEA-EDA) as synthetic substitutes for amniotic membrane potentially able to release limbal cells to the cornea.

Three samples with a different molar ratio (*X*) between PHEA-EDA and HA, have been investigated. Their swelling ability, hydrolytic resistance in simulated physiological fluid and cell compatibility (by using human dermal fibroblasts as model cell line) have been evaluated. Cell adhesion studies have been performed by using immortalized cells, such as human corneal epithelial cells (HCEC) or primary cells, such as rabbit limbal epithelial cells (RLEC) and/or rabbit limbal fibroblasts (RLF) and results have been compared to collagen gel, chosen as a positive control. Finally, the inner surface of commercial contact lenses was coated by a thin layer of each HA/PHEA-EDA hydrogel and these systems have been investigated for their ability to allow adhesion and subsequent release of limbal cells.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade, unless otherwise stated. 1-Ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium salt (NHSS) were obtained from Fluka (Milano, Italy). Bovine collagen was obtained from Fluka (UK). Fibronectin, Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), glacial acetic acid, trypsin–EDTA solution, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue solution, amphotericin B solution, penicillin–streptomycin, dispase, and fetal bovine serum (FBS) were obtained from Sigma–Aldrich (UK). Epilife medium and human cornea growth supplement were obtained from Cascade Biologics, UK. DAPI was obtained from Vector Laboratories Inc., USA. CellTracker™ Red CMTPX was obtained from Invitrogen Ltd., UK.

2.2. Preparation of HA/PHEA-EDA films

HA and PHEA-EDA were prepared and purified as described previously (Pitarresi et al., 2008; Giammona et al., 1987; Licciardi et al., 2006).

Briefly, HA with a low weight-average molecular weight was prepared by acidic degradation, as reported by Shu et al. (2002). Two stock solutions of HA (3.75%, w/v) and PHEA-EDA (6.1%, w/v) were prepared by dissolving each polymer in a 1% (w/v) NaCl aqueous solution and the pH of each solution was adjusted to 7.4 using NaOH 0.01 M. Then, 5.2 ml of the HA stock solution were mixed to adequate volumes of PHEA-EDA stock solution to obtain three different values of *X* (*X* = 0.5, 1.0 and 1.5), where *X* = moles of ethylendiamino groups of PHEA-EDA/moles of HA repeating units. The final HA concentration in each gel forming solution was 3% (w/v). After vortexing, adequate volumes of EDC aqueous solution and NHSS aqueous solution were added to the polymer mixture, in order to obtain a molar ratio of 1:1 between the EDC (or NHSS) and ethylendiamino groups of PHEA-EDA. After vigorous vortexing, the gel forming solutions were poured into Petri dishes (5.1 cm in diameter) and placed in an incubator at 37 °C for 5 days, to allow the solvent evaporation.

In this way, three different HA/PHEA-EDA films were obtained; subsequently they were exhaustively washed with twice distilled water. After drying at room temperature, the HA/PHEA-EDA films were weighed to calculate the yield and characterized (Pitarresi et al., 2008).

2.3. Equilibrium swelling studies

The equilibrium-swelling ratio of each HA/PHEA-EDA film (*X* = 0.5, 1.0 or 1.5) was determined by weight. Samples of 6 mm in diameter were immersed at 37 °C into 5.0 ml of DPBS pH 7.4 for 48 h (time enough to reach equilibrium swelling as demonstrated by dynamical swelling studies). After this time, the excess of liquid was removed with blotting paper and swollen films were weighed. Then, they were washed several times using twice distilled water to remove entrapped salts, dried by freeze-drying and finally weighed. The equilibrium swelling ratio has been expressed as *q* value (weight of swollen sample/weight of dried sample). Each experiment was performed in triplicate and results have been reported as mean value ± standard error.

2.4. Chemical hydrolysis studies

Each HA/PHEA-EDA film (*X* = 0.5, 1.0 or 1.5) of 6 mm in diameter was immersed into 5 ml of DPBS pH 7.4 and incubated in an orbital shaker (150 rpm) at 37 °C for 30 days. The hydrolytic degradation of the hydrogel film was evaluated throughout this period using the colorimetric carbazole assay (Bitter and Muir, 1962), that allows the determination of the concentration of D-glucuronic acid free units, produced from the hydrolysis of HA. Each experiment was performed in triplicate.

2.5. Preparation of collagen gel

Collagen was dissolved in 0.1 M acetic acid to a final concentration of 5 mg/ml. The solution was stirred overnight and stored at 4 °C. By diluting with DMEM, 5.7 ml of collagen solution of concentration 2.7 mg/ml were prepared; the pH was adjusted to 7, then 0.9 ml of fetal calf serum (FCS) were added. 300 μl of the final solution were put in each well of a 24-well culture plate (Corning Incorporated, USA). The plate was incubated at 37 °C for 20 min to ensure the formation of a gel.

2.6. Cell cultures

2.6.1. Human dermal fibroblasts

Following a method published previously (Ralston et al., 1999), human dermal fibroblasts (HDF) were harvested from split thickness skin grafts (STSGs) obtained from specimens following routine breast reductions and abdominoplasties. All patients gave informed consent for skin not used as part of their treatment to be used for research in a protocol approved by the Ethical Committee of the Sheffield Hospitals. All studies were performed under a HTA tissue bank licence 12179.

Fibroblasts were cultured in standard fibroblast medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2×10^{-3} mol/l glutamine, 0.625 mg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin (10% DMEM). Cells were used between passages 4 and 9.

2.6.2. Human corneal epithelial cells (HCEC)

Immortalized human corneal epithelial cells (HCEC) were obtained from LGC Promochem, UK. Cells were cultured on tissue culture polystyrene (TCP) plates coated with a solution of bovine serum albumin (0.01 mg/ml), fibronectin (0.01 mg/ml) and collagen I (0.03 mg/ml) in Epilife medium containing a human corneal growth supplement. Cells used in these experiments were between passages 45 and 47.

2.6.3. Rabbit limbal cells

Primary rabbit limbal epithelial cells (RLEC) and rabbit limbal fibroblasts (RLF) were isolated as described previously (Deshpande et al., 2009). Briefly, excised rabbit eyes obtained from Woldsway Foods Ltd., Spilsby were disinfected using 3% betadine (Medlock Medical, Oldham, UK). Limbal rims were excised from the eyes and were incubated in 2 mg/ml Dispase II solution for 2 h at 37 °C. The epithelial cells were then gently scraped with blunt forceps from the limbal tissue into PBS. The cell suspension was centrifuged and resuspended in Green's medium (DMEM) and Ham's F12 medium in a 3:1 (v/v) ratio supplemented with 10% (v/v) fetal calf serum (FCS), 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 10^{-10} mol/l cholera toxin, 1.8×10^{-4} mol/l adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 2×10^{-3} mol/l glutamine, 2×10^{-7} mol/l triiodothyrionine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were seeded into a T25 flask containing growth-arrested 3T3 feeder cells and incubated at 37 °C. The remaining limbal tissue after epithelial cell isolation was placed in T25 flasks (with 10% DMEM) to allow the explant of the limbal fibroblasts.

2.7. Cell compatibility studies

Each HA/PHEA-EDA film ($X = 0.5$, 1.0 or 1.5) was placed into a ThinCert™ PET tissue culture insert of 8 μm pore size (Greiner, UK) and each insert was placed into the well containing the cell culture medium. Human dermal fibroblasts, chosen as a model cell line, were seeded (7×10^4 cells per well) onto the surface of each film (to allow the direct contact with the sample), or at the bottom of the well. In the latter case, HA/PHEA-EDA film is not in contact with cells but only with the medium where cells are cultured for 7 days at 37 °C (indirect contact). Metabolic activity of fibroblasts was assessed using the thiazolyl blue tetrazolium bromide, MTT-ESTA test, following the procedure previously described by Higham et al. (2003).

Briefly, samples were washed three times in PBS pH 7.4 and then incubated with 1 ml MTT solution (0.5 mg/ml MTT in PBS) for 45 min at 37 °C. After this time, the unreacted solution was aspirated and the insoluble intracellular formazan product was dissolved and released from cells by adding 0.4 ml acidified isopropanol. About 0.15 ml of the product was transferred into a 96-well plate, and an optical density measurement was made at a wavelength of 540 nm. Cells cultured on tissue culture polystyrene (TCP) were used as a control. Each experiment was performed in triplicate.

2.8. Cell adhesion studies on collagen gel or HA/PHEA-EDA films

Human corneal epithelial cells (HCEC) were seeded at a density of 3×10^5 cells per sample, onto either HA/PHEA-EDA films or collagen gel, chosen as a positive control. Cells seeded onto the above mentioned samples, were cultured in Epilife medium and incubated for 24 h, and then they were placed at an air-liquid interface and cultured until 14 days. Cells on the HA/PHEA-EDA film or collagen gel were analysed after 3, 7 and 14 days using confocal microscopy (LSM 510, Zeiss).

Rabbit limbal epithelial cells (RLEC) and rabbit limbal fibroblasts (RLF) were seeded in mono and co-culture (1:1) at 3×10^5 cells per sample onto either HA/PHEA-EDA films or collagen gel. Samples with only RLF were cultured immersed into 10% FCS DMEM whereas samples with RLEC alone or in co-culture with RLF were cultured at an air liquid interface in Green's medium.

Epithelial cells (HCEC and RLEC) were prestained (before seeding), with CellTracker™ Red CMTPX (Invitrogen, UK) according to the manufacturer's instructions.

The collagen gel or HA/PHEA-EDA films were fixed using buffered formalin at day 3, 7 and 14. Cells on each sample were stained with DAPI and then analysed using confocal microscopy (Zeiss LSM 510 META). Each experiment was performed in triplicate.

2.9. Adhesion of cells on HA/PHEA-EDA films coating contact lenses

Bausch and Lomb SofLens®59 lenses (Hilafilcon B) were coated with each HA/PHEA-EDA hydrogel ($X = 0.5$, 1.0 or 1.5) by performing the cross-linking reaction directly on their inner concave surface in order to obtain a thin coating film. This surface was seeded with 1.0×10^5 RLEC alone as well as in the presence of RLF (3:1 ratio of epithelial cells and fibroblasts), then the coated lenses were put into each well of a 12-well plate and submerged in 10% Green's medium prior to be incubated at 37 °C. RLEC and RLF were pre-stained with CellTracker™ Red and CellTracker™ Green (Invitrogen, Paisley, UK) respectively before seeding according to the manufacturer's protocol. Attachment of the cells after 1, 2 and 3 days of contact with coated lenses was analysed using confocal microscopy (Zeiss LSM 510 META). Uncoated lenses were used as negative control. Each experiment was performed in triplicate.

2.10. Viability of cells released from HA/PHEA-EDA film coating contact lens

The viability of cells released in the culture medium from each hydrogel film coating contact lens, was demonstrated by MTT-ESTA assay. In particular, since after three days of incubation, few or no cell remained attached on the hydrogel, the lenses were removed from each well, the medium was changed with 1 ml of MTT solution (0.5 mg/ml MTT in PBS) and the well-plate was incubated for 45 min at 37 °C. The viability of cells released from each hydrogel film was confirmed by the presence of blue formazan crystals at the bottom of the culture well.

3. Results and discussion

The aim of this work was to evaluate the potential use of HA/PHEA-EDA films as coating materials for contact lenses able to release limbal cells as an alternative to the amniotic membrane currently employed for this purpose.

In Fig. 1 a particular of the chemical structure of HA/PHEA-EDA network is depicted to evidence the type of cross-linked bonds, i.e. amide bonds. More specifically, the cross-linking between a single PHEA-EDA chain and a single HA chain (intra-chain cross-linking) is shown. Of course, PHEA-EDA forms also cross-linked amide bonds with other HA chains (inter-chain cross-linking), that are not indicated here.

Three hydrogels, shaped as films, were obtained by varying the molar ratio (X value, equal to 0.5, 1.0 and 1.5) between the polyaspartamide derivative and the polysaccharide (see Section 2) in order to evaluate the effect of different amounts of starting polymers on some properties of these materials, such as swelling behaviour, hydrolytic resistance, cell compatibility and ability to allow adhesion and release of limbal cells.

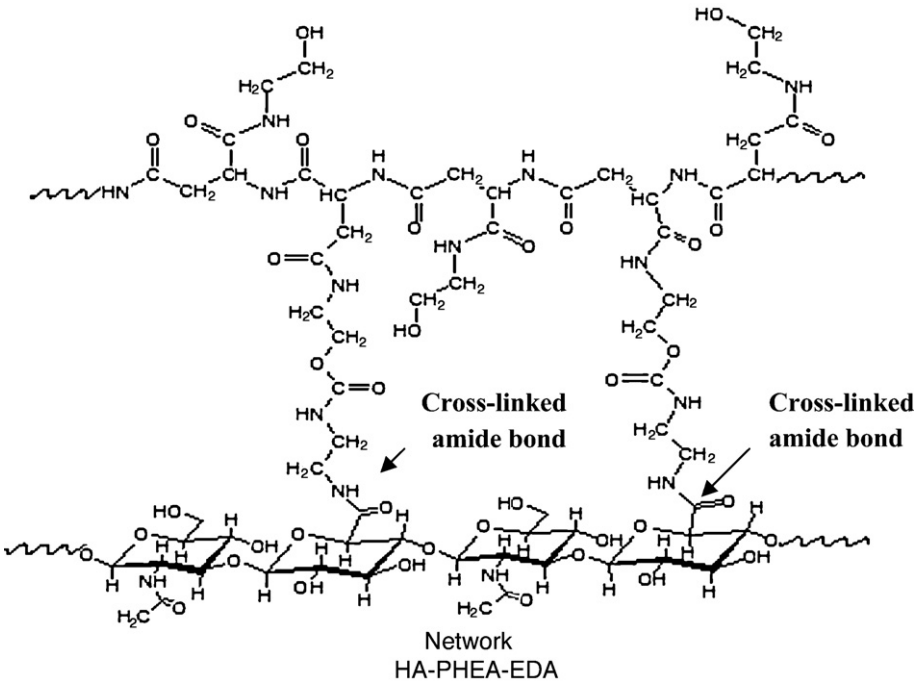

Fig. 1. Schematic representation of HA/PHEA-EDA network showing cross-linking between PHEA-EDA and a single HA chain (intra-chain cross-linking). Similarly, PHEA-EDA forms cross-linked amide bonds with other HA chains (inter-chain cross-linking).

3.1. Swelling, hydrolytic resistance and cell compatibility of HA/PHEA-EDA films

Swelling measurements performed in DPBS pH 7.4, showed that the *q* value decreases as *X* increases (see Table 1) thus confirming, as expected, that the increase in the amount of PHEA-EDA gives rise to a more compact network due to a higher cross-linking density.

In addition, these films show a long-term resistance to hydrolytic degradation in simulated physiological fluid. In fact after 30 days of incubation in DPBS pH 7.4, less than 5% of each film was lost by degradation.

Even if HA and PHEA-EDA are biocompatible polymers, cell compatibility of HA/PHEA-EDA films was evaluated to demonstrate that the cross-linked structure does not cause negative effects on metabolic activity of cells. With this aim in mind, the effect of direct and indirect contact between human dermal fibroblasts and HA/PHEA-EDA films was evaluated after 7 days of incubation. It is evident, as shown in Fig. 2, that all HA/PHEA-EDA films do not interfere in a negative manner with the cell viability.

3.2. Adhesion of cells on collagen gel or HA/PHEA-EDA films

Adhesion studies were performed using both immortalized human cells (HCEC) and primary rabbit cells (RLEC and/or RLF) in order to evaluate if adhesion depends on the choice of cells, and whether the immortalized cell line could be useful as a model to substitute primary cells. It is not unusual that the attachment of immortalized cell lines differs markedly from that of primary cells. This implies that in the development of biomaterials for clinical use it is important to work with primary cells, unless it can be demonstrated that the attachment characteristics of the chosen cell line are similar to those of the primary cells.

In addition, due to the scarcity of human corneas for research, primary rabbit limbal cells were used rather than human limbal cells.

In Table 2 a summary of the adhesion behaviour of the cells on collagen gel (as a positive control) or HA/PHEA-EDA films throughout 14 days is reported.

It is evident that HCEC adhere very well to collagen gel (see Table 2 and Panel A in Fig. 3) whereas in the presence of primary rabbit cells (RLEC, RLF) a moderate attachment occurs (see Table 2 and images A–F of Panel B in Fig. 3). Rabbit limbal fibroblasts in co-culture with rabbit limbal epithelial cells improve the adhesion on collagen gel in comparison with epithelial cells alone (see Table 2 and images G and H of Panel B in Fig. 3). In any case cell viability was maintained on the collagen gel surface from days 3 to 14 of culture.

As far as HCEC adhesion on HA/PHEA-EDA films is concerned, Table 2 shows that it depends on *X* value. In particular, a moderate adhesion is observed for *X* = 0.5 until 7 days, then it decreases after 14 days, whereas for *X* = 1.0 the adhesion continues for 14

Table 1
Equilibrium swelling ratio of HA/PHEA-EDA films, expressed as q value, after 48 h in PBS pH 7.4 at 37 °C. Values are mean ± standard error (*n* = 3).

X value	*q*
0.5	15.4 ± 0.8
1.0	8.5 ± 0.2
1.5	5.3 ± 0.4

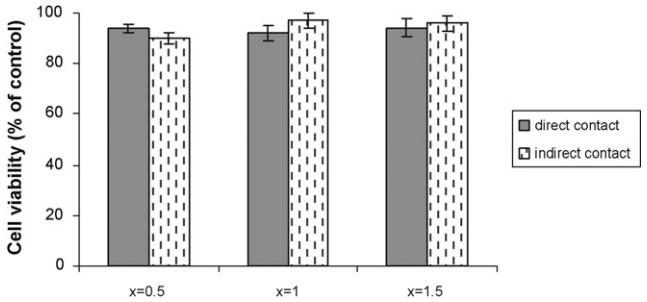

Fig. 2. Viability (% of control) of human dermal fibroblasts, kept in direct or indirect contact with HA/PHEA-EDA films (X = 0.5, 1.0 and 1.5) for 7 days, determined with MTT-ESTA test. Cells cultured on tissue culture polystyrene were used as a control. Experiments were preformed in triplicate.

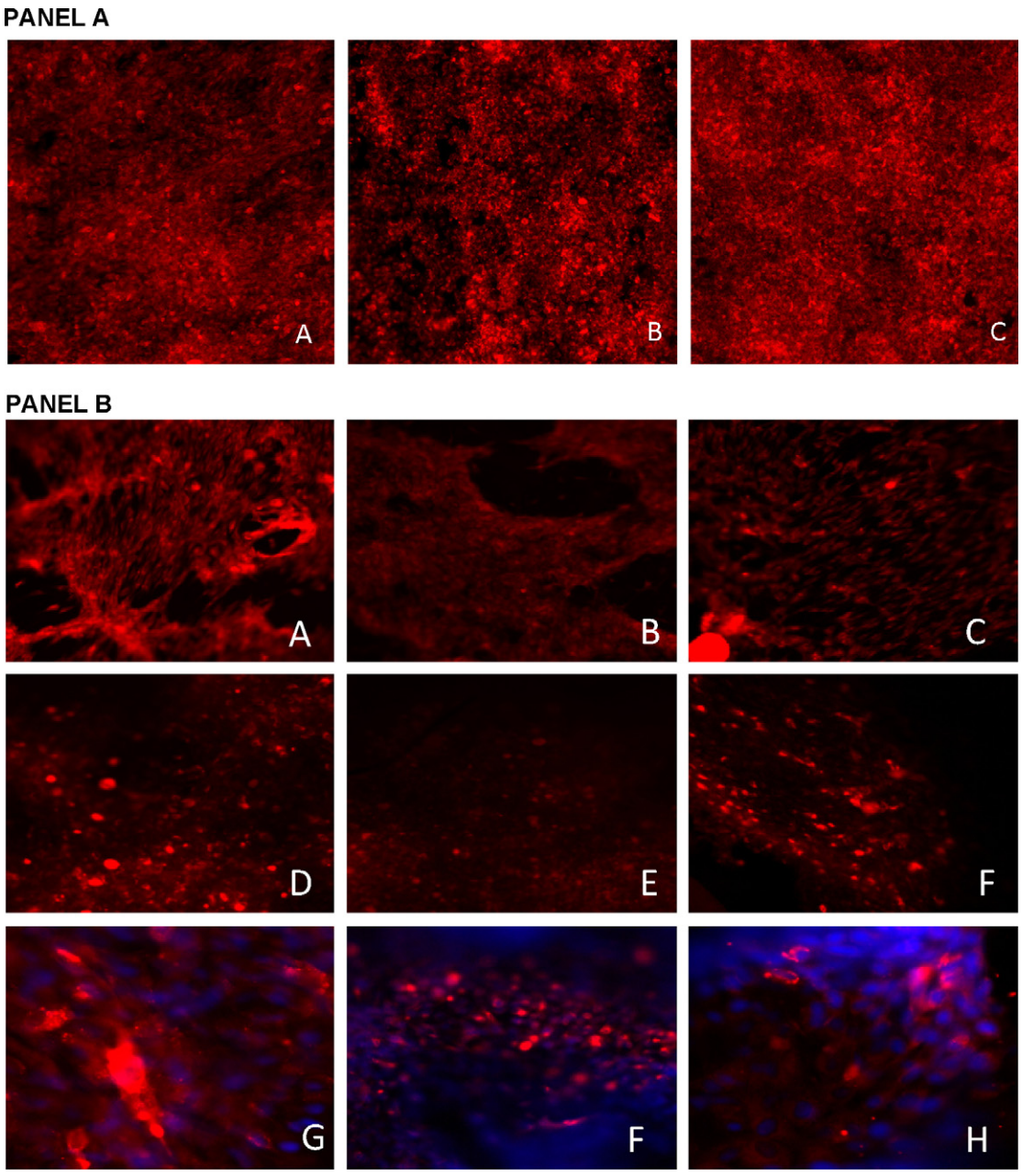

Fig. 3. Confocal images of cells in contact with collagen gel. **PANEL A**: HCEC at day 3 (A), day 7 (B) and day 14 (C) – **PANEL B**: RLF (A, B, C), RLEC (D, E, F) and co-culture RLEC (red) + RLF (blue) (G, F, H) at day 3 (A, D, G), day 7 (B, E, F) and day 14 (C, F, H). Images are representative of experiments performed in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

days. This result agrees with the increase in amino groups in the films by increasing X value (i.e. the amount of PHEA-EDA used in the cross-linking reaction), in fact it is known that the presence of amino groups on the surface of a hydrogel is a strategy to improve cell attachment (Pokharna et al., 1990; Rimmer et al., 2007). An apparently anomalous behaviour was obtained for $X = 1.5$ in fact a poor adhesion occurs until 7 days and only after 14 days a moderate attachment happens. Probably by increasing the cross-linking density (as suggested by equilibrium swelling data reported in Table 1), the amino groups in the film with $X = 1.5$ may be entrapped in the network structure thus requiring a prolonged contact time to allow cell adhesion. The results obtained show that the best sample is HA/PHEA-EDA film with $X = 1.0$ where an optimal combination between amount of amino groups and swelling ability allows cell adhesion. For this reason, in Fig. 4 we showed confocal images of HCEC adhesion only for the film with $X = 1.0$ (images A, B and C of Fig. 4).

As far as the adhesion of primary rabbit cells on HA/PHEA-EDA films is concerned, it is evident, from data reported in Table 2, that a poor adhesion occurs for RLEC and/or RLF, and only for the sample with $X = 1.0$ after 3 days of incubation a moderate attachment occurs when epithelial cells are in co-culture with fibroblasts (see image F compared with image D and E of Fig. 4). However, this attachment is not sustained, in fact after 7 and 14 days, adhesion is poor even for cells in co-culture. Results obtained from adhesion studies show that the behaviour of the immortalized human cell line (HCEC) is different from that shown by primary rabbit cells, therefore these studies suggest that the HCEC line does not represent a useful cell model to substitute primary cells in adhesion studies.

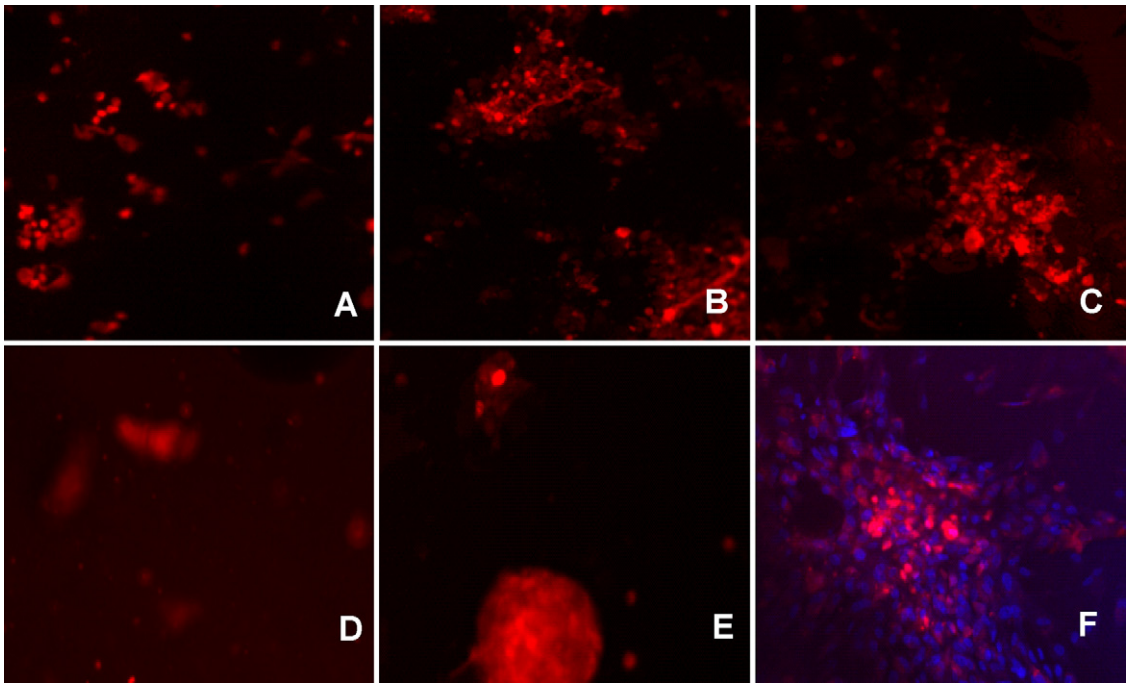

Fig. 4. Confocal images of cells in contact with HA/PHEA-EDA film with X = 1.0: HCEC at day 3 (A), day 7 (B) and day 14 (C); RLF at day 3 (D), RLEC at day 3 (E) and co-culture of RLEC (red) + RLF (blue) at day 3 (F). Images are representative of experiments performed in triplicate. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

For this reason, further studies were performed using primary rabbit cells.

3.3. Adhesion of cells on HA/PHEA-EDA films coating contact lenses

The poor/moderate cell adhesion allowed by HA-PHEA/EDA films is not a negative result, i.e. a disadvantage, but on the contrary it suggests that the investigated hydrogels could act as cell delivery systems. In fact, at this aim, cells should not strongly adhere on HA/PHEA-EDA films but they should be released from these films to the cornea.

Table 2
Adhesion of cells on collagen gel or HA/PHEA-EDA films.

Cells	Substrate	Adhesion		
		Day 3	Day 7	Day 14
HCEC	Collagen	+++	+++	+++
RLEC	Collagen	+	+	+
RLF	Collagen	+	+	+
RLEC + RLF	Collagen	++	++	++
HCEC	HA/PHEA-EDA ($X = 0.5$)	+	+	–
HCEC	HA/PHEA-EDA ($X = 1$)	+	+	+
HCEC	HA/PHEA-EDA ($X = 1.5$)	–	–	+
RLEC	HA/PHEA-EDA ($X = 0.5$)	–	–	–
RLF	HA/PHEA-EDA ($X = 0.5$)	–	–	–
RLEC + RLF	HA/PHEA-EDA ($X = 0.5$)	–	–	–
RLEC	HA/PHEA-EDA ($X = 1$)	–	–	–
RLF	HA/PHEA-EDA ($X = 1$)	–	–	–
RLEC + RLF	HA/PHEA-EDA ($X = 1$)	+	–	–
RLEC	HA/PHEA-EDA ($X = 1.5$)	–	–	–
RLF	HA/PHEA-EDA ($X = 1.5$)	–	–	–
RLEC + RLF	HA/PHEA-EDA ($X = 1.5$)	–	–	–

(–) poor adhesion; (+) moderate adhesion; (++) good adhesion; (+++) maximum adhesion. HCEC, human corneal epithelial cells; RLEC, rabbit limbal epithelial cells; RLF, rabbit limbal fibroblasts.

Moreover, the long term resistance to hydrolytic degradation in physiological conditions represents another important property: HA/PHEA-EDA films could act besides cell delivering systems, also as a barrier protective for damaged cornea, for more than 30 days.

To verify the potential ability of HA/PHEA-EDA films to release primary cells on the cornea, RLEC and/or RLF were seeded on films that coated the inner surface of commercial soft contact lenses and their adhesion was evaluated by confocal microscopy as a function of time.

In particular, the cross-linking reaction between HA and PHEA-EDA was performed directly on the inner surface of each lens in order to obtain a thin coating film. Then, this surface was seeded with RLEC alone or in co-culture with RLF and incubated until 3 days. All the experiments were performed under sterile conditions. To visualize the cells on the coated or uncoated lenses, they were stained with CellTracker™.

Initial results showed that, as expected, RLEC did not adhere on the uncoated contact lenses (data not shown) whereas a moderate adhesion occurred, for cells cultured for 3 days on the lenses coated by films with $X = 0.5$, 1.0 and 1.5 (see images A, B, C of Fig. 5). This behaviour was different from that obtained when RLEC were cultured on hydrogels alone as reported in Table 2, in fact in these conditions a poor adhesion occurs.

When RLEC were in co-culture with RLF, a greater adhesion occured for all coated lens (see images D, E, F of Fig. 5) even if the cells appeared to be clumped together.

The greater adhesion of cells on hydrogel films coating contact lenses with respect to that observed for cells on the HA/PHEA-EDA films in air/liquid culture, as well as the formation of cell clusters, in the former case, could be explained by the shifting from one-dimensional culture conditions to the three-dimensional environment represented by the coated lenses. In any case, this adhesion is only transitory, in fact after three days few or no cells remained attached on the hydrogel (images not shown) because they were released in the culture medium and their viability has been confirmed by MTT-ESTA assay (see Section 2).

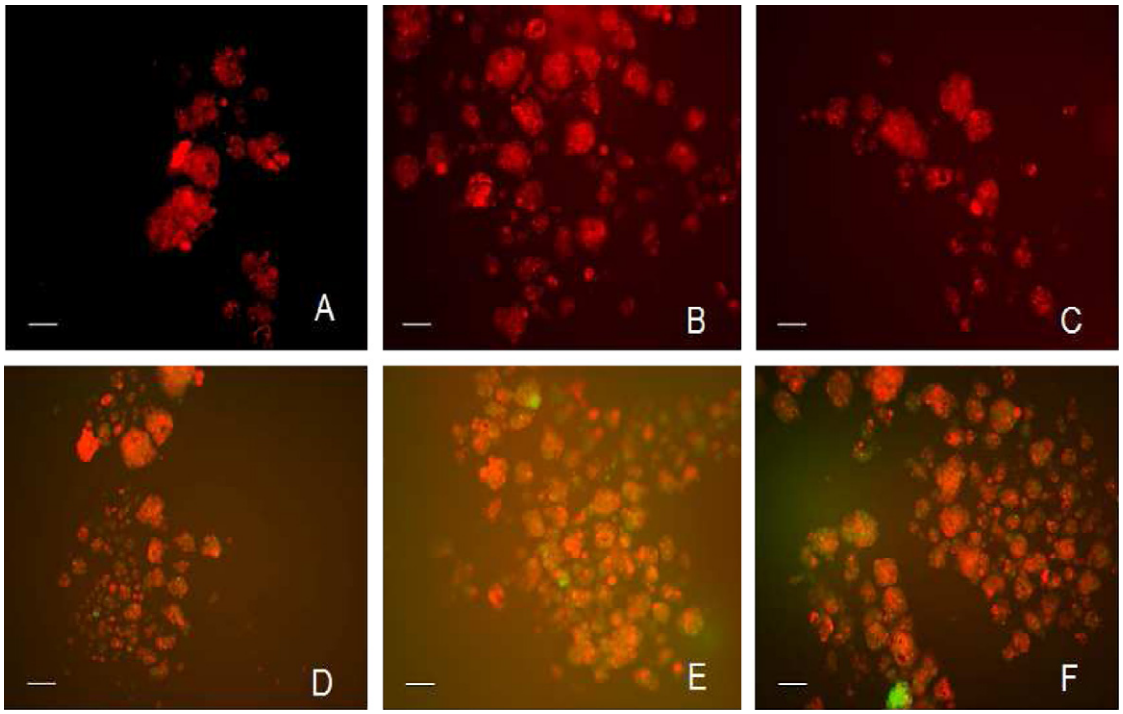

Fig. 5. Confocal images of RLEC (A, B, C) and co-culture of RLEC (red) + RLF (green) (D, E, F) after 3 days of culture on each HA/PHEA-EDA film coating the inner surface of a contact lens: film X = 0.5 (A, D), film X = 1.0 (B, E) and film X = 1.5 (C, F). Images are representative of experiments performed in triplicate. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Therefore, cells adhere on the HA/PHEA-EDA film coating the inner surface of lens, and after three days they are released, maintaining their viability, in the culture medium (and potentially they could be released in a damaged cornea).

4. Conclusion

In the present study novel biocompatible hydrogel films based on hyaluronic acid (HA) cross-linked with a polyaspartamide derivative (PHEA-EDA) have been prepared and used to coat the inner surface of commercial contact lenses. These films were able to allow the adhesion of primary rabbit limbal cells until 3 days. After this time, viable cells were released from the hydrogel surface, thus suggesting that these coated lenses could be useful for delivering limbal cells to damaged cornea as an alternative to the amniotic membrane. In particular, HA/PHEA-EDA films offer various advantages respect to the use of human amniotic membrane commonly employed for delivering limbal cells to cornea, such as: (i) absence of risk of infections (thanks to their synthetic nature), (ii) long term resistance to degradation and as a consequence the potential ability to act as protective barrier for damaged cornea, and (iii) possibility to prepare a medical device easy to be applied in the eye, i.e. a soft commercial contact lens, with the inner surface covered by a film of HA/PHEA-EDA able to be loaded with limbal cells and to release them to the cornea.

All these preliminary results encourage to continue the investigation on the prepared systems and in a further work a careful study will be performed by using a damaged cornea model.

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