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Alginate microsphere-collagen composite hydrogel for ocular drug delivery and implantation

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Abstract A composite collagen hydrogel containing protein encapsulated alginate microspheres was developed for ocular applications. Bovine serum albumin (BSA) served as a drug model. The composite hydrogel retained optical clarity and mechanical robustness of control hydrogels without microspheres. A sustained release of BSA was achieved during an 11-day period in neutral phosphate buffer. The composite hydrogel supported human corneal epithelial cell growth and had adequate mechanical strength and excellent optical clarity for possible use as therapeutic lens for drug delivery and/or use as corneal substitute for transplantation into patients who have corneal diseases.

1 Introduction

For corneal diseases, the common drug treatment is topically delivery of eye-drops, suspensions or ointments into the lower cul-de-sac. Topically delivery through eye-drops accounts for 90% of all ophthalmic formulations, which is very insufficient and in some cases leads to side effects [1, 2]. The biological barriers, mainly tear film and epithelium, limit the drug absorption and cause immediate drug loss from the pre-corneal area after topical instillation. At such, only about 5% of topically applied drugs penetrate the cornea and reach the intraocular tissue [3]. To improve ocular drug absorption, basically there are mainly two strategies: to enhance drug corneal permeability and to prolong the drug contact time on the corneal surface [4].

Drug penetration enhancers have been tested and shown to improve ocular drug absorption. However, most enhancers themselves can penetrate into the eye and cause irritation with unknown toxicological implications, limiting practical application [5].

Increasing drug contact time is another strategy for improving the efficiency of ocular drug absorption. To date, most topically instilled drugs are lost in the first 15–30 s after instillation due to tear refluxing and drainage via the nasolacrimal duct [5]. Ocular bioadhesives such as carboxymethylcellulose, hydroxypropyl cellulose, poly(-acrylic acid), Carbopol, Polycarbophil and sodium alginate in ophthalmic products have shown improved drug retention when drug was delivered topically [6]. Colloidal carriers including nanoparticles, liposomes, niosomes, microemulsions have also shown increased drug retention at ocular surface [4]. However, due to tear refluxing and drainage, mucoadhesive polymers and colloidal carriers only have limited improvement on drug residence time.

Therapeutic lens or bandage lens have been studied as potential carriers for continuous ocular drug delivery. However, these hydrated soft contact lenses equilibrated with drugs were nearly devoid of drug after only approximate 1–2 h [7, 8]. Therefore they are not ideal carriers for sustained ocular drug delivery. Collagen shield [9] formulated with drugs or absorbed with drugs could maintain on the eye for 24–48 h and maximum 72 h before melting. A number of ocular drugs such as antibacterial agents, anti-inflammatory

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agents, anti-coagulant agent, immunosuppressive agent, and antiviral agent have been studied with collagen shield as a sustained drug delivery carrier. Although used clinically to aid wound healing and delivery a variety of medication to the eyes after cataract surgery, corneal transplantation and other invasive procedures, collagen shield has several disadvantages that limited its widespread use. Collagen shield is non-transparent and is only retained for 24–72 h. It has to be applied in a physician's office and only a limited quantity of drug could be delivered.

In recent years, nanoparticles and microparticles have been extensively studied as drug delivery vehicles for sustained drug release [10]. For ocular drug delivery, studies on nanoparticles and microparticles are mainly focused on topical application for improving drug residence time [11]. Recently, a hydrogel contact lens made of poly-2-hydroxyethyl methacrylate (pHEMA) impregnated with lidocaine-loaded silica nanoparticles was fabricated and showed sustainable release of lidocaine over 8 days [12]. This system allowed for thermodynamically stable microemulsions and suitable for encapsulating hydrophobic drugs, but unfavorable for encapsulation of hydrophilic drugs. Moreover, the high temperature (60°C) needed for drug encapsulation and hydrogel fabrication makes this system unsuitable for encapsulation of bioactive factors that are sensitive to high temperatures.

Alginate is a biocompatible polysaccharide that has been extensively studied as matrices for cell and growth factor encapsulation, and also for gene delivery due to its fast ion-inducible gelation under mild condition without involvement of organic solvents [13, 14]. Furthermore, the size of alginate microspheres can be tuned by varying the air-flow of droplet generator. In situ formed gels from alginate or its composite with pluronic or HPMC loaded with pilocarpine [15] or gatifloxacin [16] have shown improved drug bioavailability.

In our previous work, we have prepared optically clear and mechanically strong collagen-based hydrogels as corneal substitutes that have been successfully tested in pig model showing stable host-graft integration and promoting corneal cell and nerve regeneration [17]. The objective of this study was to incorporate drug-loaded microparticles into collagen hydrogel to simultaneously deliver drugs or bioactive factors during transplantation when the resulting composite hydrogel is used as corneal substitute or to provide sustained drug release when used as therapeutic lens. We demonstrated successful fabrication of composite collagen hydrogels containing alginate microspheres as carriers using bovine serum albumin (BSA) as a model protein for studying release characteristics. The mechanical and optical properties as well as the in vitro compatibility of the composite hydrogel to human corneal epithelial cells were also studied.

2 Materials and methods

2.1 Materials

Porcine type I atelocollagen was purchased from Nippon Meat Packers Inc. (Tokyo, Japan). Morpholinoethanesulfonic acid (MES; EMD Chemicals Inc. USA) was dissolved in deionized water to form a 0.62 M MES buffer solution. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were supplied by Fluka (Buchs, Switzerland). Intermediate-G sodium alginate, bovine serum albumin (BSA) and fluorescein-labeled BSA (FITC-BSA) were obtained from Sigma–Aldrich (Oakville, Ontario, Canada). Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, California).

All other reagents were of analytical grade and used as received.

2.2 Preparation of BSA-loaded alginate microspheres

Ten milligram of BSA was dissolved in 3 ml of 1.5 w/v% alginate solution and was gently shaken for 10 min. The mixture was drawn into a 1 ml syringe and ejected at a constant nozzle speed through a 26G 1/2 needle by a microdroplet generator. The resulting microdroplets were converted into alginate microspheres by gelation in a 2% $CaCl_2$ solution for 15 min. The microspheres were collected by centrifugation at 5,000 rpm for 8 min (Beckmann Avanti 30, Beckmann, USA) followed by washing with phosphate buffered saline (PBS). After two cycles of washing and centrifugation, the micropsheres obtained were redispersed in 1.2 ml of water and used right away for microsphere-collagen composite hydrogel preparation.

BSA loading efficiency (LE) in microspheres was estimated in terms of the following formula:

$$\mathrm{LE} = (W_0 - W_\mathrm{f})/W_0 \times 100\%$$

where W_0 is the total amount of BSA fed into the alginate solution and W_f is the total amount of BSA in the supernatant after gelation. Calculations were done upon three replicates. The concentration of BSA in supernatant was quantified via Bio-Rad protocol using DU® 640 spectrophotometer.

2.3 Preparation of alginate microspheres-embedded collagen composite hydrogels

0.3 ml of 13.7 wt% collagen and 0.1 ml of MES (0.62 M) were mixed in a syringe mixing system we previously reported [17, 18], followed by addition of 0.6 ml of the above alginate microspheres. After a homogenous solution was formed, 57 μl of EDC/NHS solution was injected and mixed into the mixture in a molar equivalent ratio of EDC:

NHS: collagen amine groups at 3:3:1. The mixture was cured at room temperature with 100% humidity for 16 h, followed by post-curing at 37°C for 5 h. The resulting composite hydrogel was coded as Coll-Alg-0.6. In the same way, composite hydrogels with 0.1 and 0.3 ml of alginate microspheres added into collagen solutions were made except for respective supplement of 0.5 and 0.3 ml of MES solution to ensure the same total volume as that of Coll-Alg-0.6 prior to gelation, and the composite hydrogels were coded as Coll-Alg-0.1 and Coll-Alg-0.3, respectively.

2.4 Morphological and optical properties

Refractive indices of PBS equilibrated flat hydrogel films (~50 μm thick) were recorded on a VEE GEE refractometer at 21°C with bromonaphthalene as the calibration agent.

White light (quartz-halogen lamp source) transmission and backscatter measurements of cornea-shaped hydrogels were performed on a custom-built instrument [19].

The morphology of composite hydrogels was observed using a light microscope (Axiovert S100TV, Germany) with an attached digital camera. The particle size was estimated from the measurement of a hundred of particles found in a randomly chosen area in the pictures.

Confocal microscopic imaging of FITC-BSA encapsulated alginate microspheres and the micropshere-collagen composite hydrogel were carried out using a confocal laser scanning microscopy (LSM 510 meta, Axiovert 100, Zeiss, Germany). Fluorescein was excited by a 488 nm argon laser and the emission was observed at 512 nm.

2.5 Mechanical properties

The tensile strength, elongation at break, and elastic moduli of the hydrogels were determined on an Instron's electromechanical universal tester (Model 3340, Instron, Norwood, MA) equipped with a Series IX/S software. Flat hydrogels, 0.50 mm thick, were equilibrated in PBS and cut into 12 mm × 5 mm rectangular sheets. To enhance the gripping of the clips and prevent damage of the specimen from clipping, two ends of each specimen were glued to a mounting tape using tissue adhesive, Dermabond (Ethicon Inc., Somerville, NJ). The actual gauge length of each specimen is 5 mm for testing. Three specimens were measured for each hydrogel formulation. The crosshead speed was at 10 mm min^{-1}.

2.6 Equilibrated water content

After removal from the molds, composite hydrogels were immersed in PBS for 7 days at 4°C and 6 h at room temperature. The hydrogels were taken out of the PBS and the surface was gently blotted with filter paper, after which the hydrogels were weighed on a microbalance to record the wet weight of the samples. These hydrogels of known weight were then dried at room temperature under vacuum until a constant weight was attained. The total equilibrated water content of hydrogels (W_t) was calculated according to the following equation:

$$W_t = (W - W_0)/W \times 100\%$$

where W_0 and W denote weights of dried and PBS equilibrated samples, respectively.

2.7 BSA release from alginate microspheres-embedded collagen hydrogels

In order to remove impurities, the composite hydrogels obtained were washed thoroughly with water and soaked in PBS for 24 h with fresh PBS replacement every 8 h. The purified samples were then immersed in 50 ml of PBS buffer at 37°C. During the controlled release process, 5 ml of aliquots of the release media was taken out, and meanwhile supplemented with 5 ml of fresh buffer at every predetermined time interval and the concentration of the BSA released was determined via Bio-Rad method on a DU® 640 spectrophotometer. The calibration curve was made using non-loaded BSA composite hydrogel as correction.

$$\text{Cumulative amount released}\,(\%) = M_t/M_\infty \times 100\%.$$

where M_t is the amount of drug released from the composite gels at time t and M_∞ is the amount of BSA preloaded in composite gels.

2.8 Epithelial cell compatibility assay

Immortalized human corneal epithelial cells (HCECs) were used to evaluate in vitro epithelial coverage, using the same method we previously described [20].

3 Results and discussion

3.1 Morphology and optical properties

The size of alginate microspheres will influence the optical properties of microsphere-collagen composite hydrogel. Our alginate microspheres have an average diameter of 25 ± 10 μm. BSA-loaded microspheres are homogeneously distributed within the composite hydrogels (Fig. 1c–e). Figure 2a and b present confocal fluorescence images of FITC-BSA-loaded free alginate microspheres and FITC-BSA loaded alginate microsphere-collagen composite hydrogel (Coll-Alg-0.3). The bright color

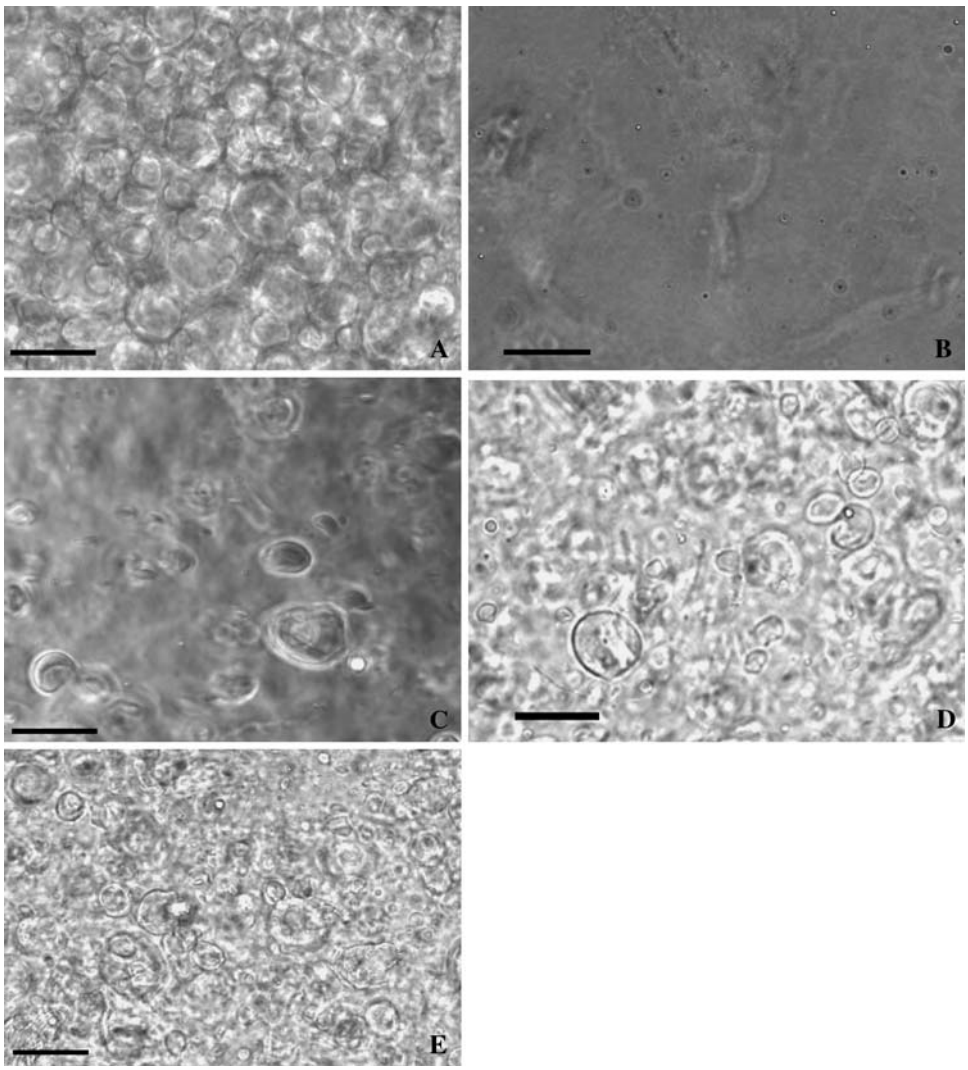

Fig. 1 Light microscope images of alginate microsheres (**a**), blank collagen gel (**b**), Coll-Alg-0.1 (**c**), Coll-Alg-0.3 (**d**) and Coll-Alg-0.6 (**e**). Bar = 50 μm

represents FITC-BSA. As indicated in the images, BSA is evenly distributed inside free alginate microspheres as well as inside those embedded throughout collagen hydrogel matrix. The alginate microparticles still remain the shape as micropsheres in the composite hydrogel (Fig. 2b).

The white light transmission of the composite hydrogels deceased as the increase of the loading of alginate microspheres (Table 1), however, the backscatter values went up accordingly but still approximately equivalent to that of human cornea (3%) [21]. The transmission value of hydrogel Coll-Alg-0.6 (84%) with the highest loading of microspheres is slightly lower than that of human cornea (87%) [22].

The encapsulation of microspheres did not significantly change the refractive indices of composite hydrogels although the refractive indices decreases as the increase of the loading of alginate microphores (Table 1). The decrease of refractive indices is due to the increase of EWC (Table 1) along with the increase of alginate microspheres (Fig. 3).

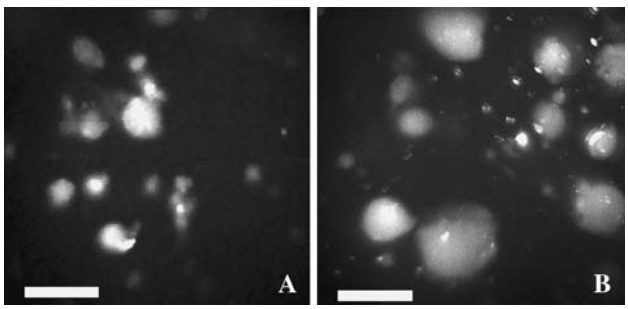

Fig. 2 Confocal images of FITC-BSA-alginate microspheres (**a**) and FITC-BSA-Coll-Alg-0.3 (**b**). Bar = 50 μm

3.2 Mechanical properties of composite hydrogel

The hydrogels should be mechanically strong enough for handling, wearing and suturing into the host. We recorded the tensile strength, break strain and elastic moduli of collagen and its composite hydrogels (Fig. 4). The data

Table 1 Optical properties of composite hydrogels[a]

Hydrogels	White light transmission (%)	Light scatter (%)	Refractive indices	EWC (%)
Collagen gel	92.0 ± 2.9	1.8 ± 0.1	1.3493 ± 0.0011	91.9 ± 0.4
Coll-Alg-0.1	87.2 ± 0.8	3.1 ± 0.8	1.3449 ± 0.0001	92.5 ± 0.5
Coll-Alg-0.3	85.5 ± 2.1	4.0 ± 0.3	1.3446 ± 0.0002	93.4 ± 0.8
Coll-Alg-0.6	84.4 ± 2.7	4.0 ± 0.7	1.3374 ± 0.0003	97.3 ± 0.4
Human cornea	87[b]	3[c]	1.373–1.380[d]	78[e]

[a] The value is denoted as mean ± standard deviation ($n = 3$ samples for each hydrogel)

[b] Ref. [22], [c] Ref. [21], [d] Ref. [23], [e] Ref. [24]

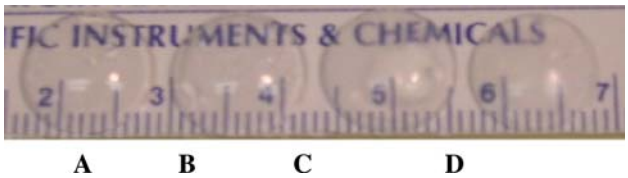

Fig. 3 Digital images of hydrogels. (**a**) Collagen hydrogel; (**b**) Coll-Alg-0.1; (**c**) Coll-Alg-0.3 and (**d**) Coll-Alg-0.6. Images **a** and **b** were reproduced from Corneal Regenerative Medicine: Corneal Substitute for Transplantation. Authors: M. Griffith, P. Fergaholm, W. Liu, C. R. McLauglin and F. Li in Essentials in Ophthalmology—Cornea and External Eye Disease. Thomas Reinhard and Frank Larkin (Eds.), Chapter 3, 2008. With kind permission of Springer Science and Business Media

show that the tensile strength and modulus of composite hydrogels increased with microsphere content at lower loadings (Coll-Alg-0.1 and Coll-Alg-0.3), while the break strain has unchanged. However the properties of hydrogel with high sphere content (Coll-Alg-0.6) are badly deteriorated with its modulus decreased approximately 10-fold relative to collagen hydrogel in spite of high break strain. A reasonable explanation is that for Coll-Alg-0.1 and Coll-Alg-0.3 composite hydrogels, the impregnated microspheres possibly act as fillings or reinforced additives to some extent in polymer composites, which aid in dissipating energy to reinforce the collagen matrix. But with high microspheres contents such as in Coll-Alg-0.6, the network becomes somewhat discontinuous with interruptions or voids, which may significantly reduces the capacity of network to bear exterior load, leading to poor mechanical properties.

3.3 BSA release kinetics

The loading efficiency of BSA in alginate microspheres was estimated to be approximately 63.6%. The loss of BSA into the soaking buffer and washing PBS was approximately 2.8%, 2.2% and 2.1% for Coll-Alg-0.6, Coll-Alg-0.3 and Coll-Alg-0.1, respectively.

In this study, we used BSA as a model drug to examine the release behavior of protein from composite hydrogels. Alginate micropsheres have been widely studied as drug delivery system. Early studies demonstrated that there was

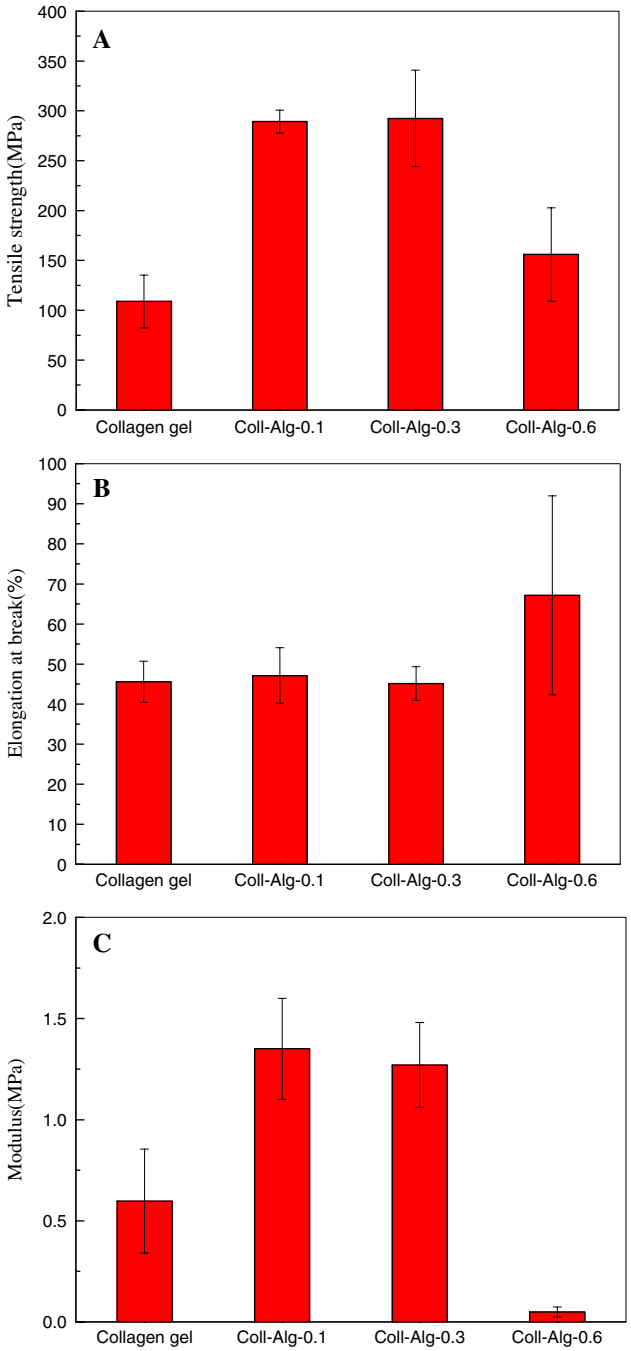

Fig. 4 Tensile strength (**a**), average break strain (**b**) and moduli (**c**) of hydrogels

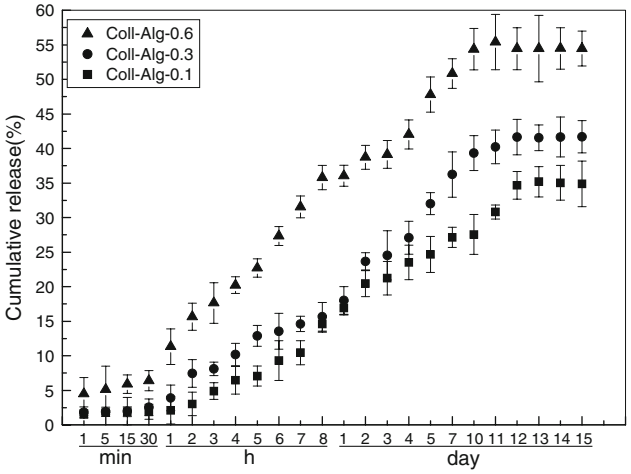

Fig. 5 BSA release from composite hydrogels. Reproduced partially from Corneal Regenerative Medicine: Corneal Substitute for Transplantation. Authors: M. Griffith, P. Fergaholm, W. Liu, C. R. McLauglin and F. Li in Essentials in Ophthalmology—Cornea and External Eye Disease. Thomas Reinhard and Frank Larkin (Eds.), Chapter 3, 2008. With kind permission of Springer Science and Business Media

a typical initial burst release of BSA from alginate-$CaCl_2$ microspheres due to the unstable nature of alginate microspheres in phosphate buffer at pH above 5 [25]. Figure 5 displays the kinetics of BSA release from three types of composite hydrogels in PBS (pH 7.4). In all cases, no initial burst release was observed. It should be noted that no microspheres were observed on the surface of collagen matrix, which means that all microspheres were embedded inside the hydrogels. Thus, in this composite hydrogel release system, BSA has to permeate through two barriers, i.e. micropshere compartment and collagen matrix, for release into solution. In spite of the burst release of BSA from alginate microspheres at beginning [25], BSA is first absorbed by collagen networks surrounding the micropsheres, and then gradually diffuses out of the matrix. This dual-barrier considerably suppresses the initial abrupt release. For all the three composite hydrogels, a similar release pattern is shown. After 1 h, the release amount is augmented till day 11; after that BSA release becomes steady again. On day 15, 34.9%, 41.7% and 54.5% BSA was released from Coll-Alg-0.1, Coll-Alg-0.3 and Coll-Alg-0.6, respectively. We intended to further track the BSA release, however, no newly released BSA was detected. A possible reason is that there exists electrostatic interaction between alginate and BSA, which prevents the rest of the BSA from permeating out. Nonetheless, a sustainable protein release can be realized by encapsulating BSA-loaded alginate microspheres into collagen hydrogel. In our future work, we will extend this strategy to the controlled release of other bioactive proteins such as nerve growth factors.

3.4 Epithelial cell compatibility

To be suitable as therapeutic lens or corneal substitute for implantation, the composite hydrogel has to be biocompatible, and non-toxic to ocular tissues. Since corneal epithelial cells will be expected to re-grow on the hydrogel when used as corneal substitute, we evaluated the growth behaviour of HCECs on a selected formulation, Coll-Alg-0.3 hydrogel. In vitro results demonstrated that HCECs became confluent at day 3 (Fig. 6). No toxicity to cells was observed. Overall, the composite hydrogel well supported the attachment and proliferation of corneal epithelial cells.

4 Conclusion

We have demonstrated that a transparent collagen-based composite hydrogel containing microspheres encapsulating drugs or bioactive factors are feasible for use in transplantation to aid in repair or regeneration process, or to confer anti-infective properties. The resulting hydrogels could also be suitable as therapeutic lenses for sustained drug release. Such composite hydrogels based on natural materials may therefore be useful in therapeutic application in the future, as biointeractive implants or as therapeutic inserts or contact lenses.

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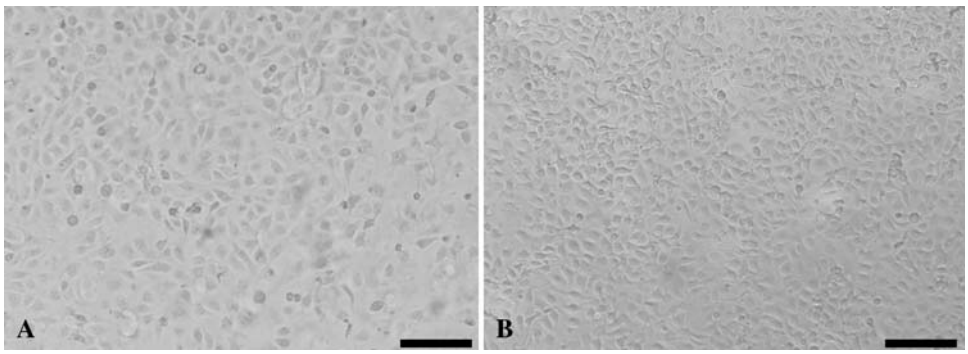

Fig. 6 HCECs overgrowth on Coll-Alg-0.3 hydrogel. (**a**) Cells at day 1 post-seeding; (**b**) Cells at day 3 post-seeding became confluent (Bar = 200 μm)

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