Chitosan-Gelatin Biopolymers as Carrier Substrata for Limbal Epithelial Stem Cells

A. De la Mata, M.Sc.,[1,2] T. Nieto-Miguel, Ph.D.,[2,1] M. Lopez-Paniagua, M.Sc.,[1,2] S. Galindo, M.Sc.,[1,2], M.R. Aguilar, Ph.D.,[3,2] L. García-Fernández, Ph.D.,[3,2] B. Vazquez, Ph.D.,[3,2] J. San Roman, Ph.D.,[3,2] R.M. Corrales, Ph.D.,[1,2,a]* M. Calonge, MD., Ph.D.[1,2]*

[1]IOBA (Institute of Applied Ophthalmobiology), University of Valladolid, Valladolid, Spain; [2]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Spain; [3]Institute of Polymer Science and Technology, Spanish National Research Council (CSIC), Madrid, Spain;

[a]Current affiliation: Ocular Surface Centre, Baylor College of Medicine, Houston, TX, USA.

*These authors contributed equally to this study and should therefore be regarded as equivalent authors.

Ana de la Mata Tel: (+34)983184761; e-mail: adelamatas@ioba.med.uva.es

Teresa Nieto-Miguel: Tel: (+34)983184754; e-mail: tnietom@ioba.med.uva.es

Marina López-Paniagua: Tel: (+34)983184761; e-mail: marina@ioba.med.uva.es

Sara Galindo: Tel: (+34)983184761; e-mail: sgalindor@ioba.med.uva.es

Maria Rosa Aguilar: Tel: (+34)915622900; e-mail: mraguilar@ictp.csic.es

Luis García-Fernández: Tel: (+34)915622900; e-mail: luisgar@ictp.csic.es

Blanca Vazquez: e-mail: Tel: (+34)915622900; bvazquez@ictp.csic.es

Julio San Román: (+34)915622900; e-mail: jsroman@ictp.csic.es

Rosa Maria Corrales (current affiliation): Tel: 713-798-8381; e-mail: corrales@bcm.edu;

Margarita Calonge: Tel: (+34)983184750; e-mail: calonge@ioba.med.uva.es

Corresponding author: M. Calonge

Telephone: (+34)983184750 E-mail: calonge@ioba.med.uva.es

Fax: (+34)983184762

Corresponding author: A. De la Mata

Telephone: (+34)983184761 E-mail: adelamatas@ioba.med.uva.es

Fax: (+34)983184762

Edificio IOBA (Instituto de Oftalmobiología Aplicada)

Campus Miguel Delibes

Paseo de Belén, 17

E-47011 Valladolid, Spain

ABSTRACT

The aim of this work was to evaluate semi-synthetic biopolymers based on chitosan (CH) and gelatin (G) as potential *in vitro* carrier substrata for human limbal epithelial cells (hLECs). To that end, human corneal epithelial cells (HCE) were cultured onto different CH-G membranes. None of the polymers were cytotoxic and cell proliferation was higher when CH was functionalized with G. Expression levels of corneal epithelial markers (K3, K12, E-caherin, desmoplakin, and zonula ocludens (ZO)-1) were better maintained in HCE cells grown on CH-G 20:80 membranes than other proportions. Consequently, CH-G 20:80 was chosen for the subsequent expansion of hLECs. Cells derived from limbal explants were successfully expanded on CH-G 20:80 membranes using a culture medium lacking components of non-human animal origin. The expression levels found for corneal (K3 and K12) and limbal epithelial stem cells (K15) specific markers were similar to or higher than those found in limbal cells grown onto the control substratum. Our results demonstrate that CH-G 20:80 membranes are suitable for the expansion and maintenance of stem cells derived from the limbal niche. These results strongly support the use of polymers as alternative substrata for the transplantation of cultivated limbal cells onto the ocular surface.

Introduction

Damage to the ocular surface is one of the main causes of blindness. The limbus, located at the corneoscleral junction, contains a population of stem cells in the basal layer of the epithelium. These limbal epithelial stem cells (LESCs) possess all of the properties of an adult stem cell population[1] and are responsible for maintaining and regenerating the corneal epithelium. LESC deficiency syndrome is characterized by the loss or dysfunction of LESCs, and usually results in corneal blindness by causing an unstable corneal epithelium, invasion of the conjunctiva onto the corneal surface, neovascularization, and persistent inflammation.[2]

Corneal transplantation is often the only option to recover vision, but a poor outcome usually occurs in the absence of viable of LESCs.[3] Restoration of the stem cell population is a possible treatment for LESC deficiency. The first attempt to transplant LESCs that were previously expanded *in vitro* was reported by Pellegrini et al. in 1997,[4] with subsequent attempts by these and other authors.[5-9] A

substratum is necessary for the *in vitro* expansion of epithelial cells and subsequent transplantation onto the ocular surface.[10, 11] For instance, fibrin,[7] human amniotic membrane (HAM),[8, 9] and cellular feeder layers such as 3T3 fibroblasts[4, 7] have facilitated the expansion of corneal epithelial cells. However as natural products, each of these agents cannot be standardized.[12-14]

As alternatives to biological materials, recent progress in tissue engineering has provided new polymeric biomaterials that can serve as substrata or scaffolds for the culture of stem cells.[15] These biomaterials could affect stem cell behavior depending on how closely they mimic the natural stem cell microenvironment or niche.[16] The novel synthetic substrata are advantageous because they provide suitable optical and mechanical characteristics for the subsequent ocular application. In addition, they might overcome the typical limitations of a biological material. To improve the current techniques for ocular surface transplantation, different biosynthetic materials have been used to expand conjunctival epithelial cells or to induce stratification of human corneal epithelial cells.[11, 17-21]

Substrata for LESC transplantation should be optically clear, robust enough to withstand manipulation and suturing, and biocompatible with minimal immunogenicity. Additionally, they should support the growth of LESCs without induction of cell differentiation to mature corneal epithelial cells.[22] Chitosan, a polysaccharide obtained from deacetylation of natural chitin, is a biocompatible, non-toxic, and bioresorbable polymer with antibacterial properties.[23, 24] Another candidate substratum for LESC expansion is gelatin, derived from type I collagen, the primary component of the extracellular matrix in the eye and the skin. It is biodegradable, resorbable, non-immunogenic under physiological conditions, and it has mechanical properties that can be modulated.[25] Therefore, chitosan and/or combinations of chitosan and gelatin could potentially provide a suitable substratum for LESC transplantation.

To find alternatives for ocular surface reconstruction, we investigated several polymers based on different concentrations of chitosan and gelatin as potential carrier substrata for LESCs. With consideration of the ultimate goal to use the methods developed by us in clinical applications, we were mindful of the potential risks in using culture media containing defined or undefined animal derivatives. Such components have the potential to transmit communicable diseases and/or provoke immunological problems during transplantation.[26] Therefore to reduce the potential harmful complications and to minimize any risk for future patients, we used a culture medium that was free of supplements containing non-human animal derivatives.

Material and Methods

Reagents

Phosphate-buffered saline solution (PBS), Hanks balanced salt solution, trypsin–ethylenediamine-tetraacetic acid (EDTA) 1X, Versene, Dulbecco's modified Eagle's medium (DMEM/F12), fetal bovine serum (FBS), epidermal growth factor (EFG), human insulin, penicillin/streptomycin, gentamicin, and fungizone were purchased from Invitrogen-Gibco (Inchinan, UK). Dimethyl sulfoxide (DMSO), pig skin gelatin type A, and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Human serum and cholera toxin were purchased from Lonza (Basel, Switzerland) and Gentaur (Kanpenhout, Belgium) respectively. The Viability-Cytotoxicity Assay Kit for Mammalian Live and Dead Cells was purchased from Biotium, Inc. (Hayward, CA, USA), and the proliferation assay kit Alamar Blue™ was purchased from AbD Serotec (Oxford, UK).

Chitosan and chitosan-gelatin membrane synthesis and physical-chemical characterization

Chitosan (85% deacetylated; INDEBIO; Salamanca, Spain) was dissolved in 0.15 M acetic acid and filtered to remove any solid impurities. Pig skin gelatin (Type A, Sigma) was added to achieve the following chitosan-gelatin (CH-G) proportions (w/w): CH-G 80:20, CH-G 50:50, and CH-G 20:80. Moreover, CH 100 and G 100 samples were also prepared by crosslinking the corresponding compound with glutaraldehyde as described in the following paragraph. The solution was gently stirred at 40ºC until complete dissolution of the components. Glutaraldehyde (0.50% by weight) was added as chemical crosslinker. Polymeric membranes were obtained by casting in 10 cm Teflon molds at 40ºC for 3 days. Samples were washed with distilled water followed by 1% w/v NaOH, and then distilled water again. They were immersed in a 0.3% $NaBH_4$ solution in ethanol for 1 h to reduce Schiff bases formed between primary amine groups during the crosslinking reactions induced by glutaraldehyde and any free non-reacted aldehyde group.[27] Finally, samples were thoroughly washed with water, cut in 1.5-cm diameter circles, and stored in an ethanol:water (70:30) solution until used.

A Perkin-Elmer TGA-7 Thermogravimetric Analyzer interfaced to a thermal analysis data system TAC7/DX was used to study the thermal stability of the polymers (Waltham, MA, USA). Thermograms were obtained under nitrogen atmosphere, between 50 and 500ºC, using a constant heating rate of 10ºC/min. Chemical composition and possible interactions between the biomolecules were determined by

measuring attenuated total reflectance Fourier-transformed infrared (FTIR) spectra with a Perkin-Elmer Spectrum One FTIR Spectometer (Waltham, MA, USA) coupled to an attenuated total reflectance device. Infrared scans were obtained between 4000 and 400 cm^{-1}, with a scanning resolution of 2 cm^{-1}. Thirty-two scans were recorded for each sample. The degree of swelling and weight loss of the polymeric membranes were monitored gravimetrically at acidic (pH 2), basic (pH 10), and physiological pH (pH 7.4) at 37ºC for 2 weeks.

Human corneal epithelial cell culture

Stainless steel rings with a 12-mm inner diameter were placed on top of the control tissue culture plastic (TCP) and on top of the polymeric membranes that were placed on the TCP. These rings delimitated an area of 1.1 cm^2 and prevented the polymer membranes from floating. Simian virus-40-transformed human corneal epithelial (HCE)[28] cells (a kind gift from Arto Urtti, University of Helsinki, Finland) were used to test different polymers as cellular substrata. A total of $4x10^4$ cells/cm^2 were seeded on the TCP and on each biopolymer. The culture medium contained DMEM/F12 supplemented with 15% FBS, 0.5% DMSO, 0.1 µg/ml cholera toxin, 10 ng/ml EGF, 5 µg/ml human insulin, and antibiotics (62.5 U/ml penicillin, 62.5 mg/ml streptomycin). The cells were incubated at 37ºC, under 5% CO_2 and 95% humidified air. The culture medium was carefully changed every 2-3 days.

Human limbal explant culture

Cadaveric human corneoscleral buttons were obtained with informed research consent from Barraquer Eye Bank of Barcelona (Spain). The average age ± standard error of the mean (SEM) of the donors was 82.9 ± 2.8 years. The buttons were stored at 4ºC and were used within 3.2 ± 0.4 days of the donor's death.

Human limbal epithelial cells were grown from limbal explants obtained from the corneoscleral buttons. Briefly, the corneoscleral tissue of 8 different donors was rinsed with Hanks balanced salt solution containing 50 µg/ml gentamicin and 2.5 µg/ml fungizone. The excess conjunctiva, iris, and corneal endothelium tissues were carefully removed, and a 7.5 mm trephine was used to isolate the central cornea from the limbus. Then, 1-2 mm^2 segments from the superior and inferior limbal rings were excised and used to establish 44 cultures. These segments contain the highest concentrations of limbal palisades of Vogt that constitute the limbal niche of stem cells.[29] The segments were placed epithelial side up at the center of stainless steel ring holding each substratum in place. After drying the explants for 30 min inside

a laminar flow hood, 50 µl of human serum was added onto the explants and incubated for 6 h to promote attachment to the substratum. After 6 h, 500 µl per 3.8 cm^2 (12-well plate area) of culture medium was added. The culture medium was based on DMEM/F12 suplemented with 10% human serum, 5 ng/ml EGF, 5 µg/ml human insulin, 0.4 µg/ml hydrocortisone, 1 µM isoproterenol, 0.18 mM adenine, 2 nM triiodothyronine, 50 µg/ml gentamicin, and 2.5 µg/ml fungizone. When cells began growing onto the substrata, the total amount of medium added was 1 ml.

All cultures were incubated at 37ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two or three days, and the cultures were monitored using a phase contrast microscope (Eclipse TS100, Nikon, Tokyo, Japan). The time when each explant started to grow and reach confluence was recorded.

Cell viability

The viability of HCE cells grown on each substratum was analyzed at 6 h and at 1, 2, 4, and 8 days. Cellular viability-cytotoxicity assays were performed on attached cells by using the Viability-Cytotoxicity Assay Kit for Mammalian Live and Dead Cells. The kit uses a combination of calcein-AM and ethidium homodimer (EthD-III) to dye live cells green and dead cells red. At each time point, the culture medium was gently removed and cells were washed with PBS. After staining according to the manufacturer's instructions, the cultures were rinsed with PBS prior to being viewed under a fluorescence microscope (Leica DMI 6000 B, Leica, Wetzlar, Germany). Live and dead cells were counted in 5 random fields per substratum and time. Viability was expressed as the percent of live cells.

Cell proliferation

Cell growth was determined by using the fluorometric non-toxic Alamar Blue™ (AB) assay, which depends on the conversion of resazurin to resorufin, a pink fluorescent dye. The reaction is based upon chemical reduction of the culture medium resulting from cell growth. The AB assay was performed after 1, 2, 4, and 8 days according to the manufacturer's instructions. After 5 h of incubation, duplicate 300 µl samples of culture medium for each test sample were transferred to a 24-well plate. Fluorescence was measured on a SpectraMAx M5 (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 560 nm and emission wavelength of 590 nm, according to the specifications of the manufacturer.

A total of $4x10^4$ cells/cm^2 were seeded onto TCP and each biopolymer. To consider only the cells adhered to the biopolymers, the polymeric membranes were transferred to another clean well. Proliferation data were determined as the relative cell density compared with the cell density at 1 day.

Real time reverse transcriptase polymerase chain reaction (RT-PCR)

Real time RT-PCR was performed to determine the expression levels of specific corneal and limbal genes (Table 1). Cytokeratins 3 (KRT3) and 12 (KRT12) are cytoskeletal markers of differentiated corneal epithelial cells.[30-32] Additionally, expression of adhesion genes like E-cadherin, desmoplakin, and zonula occludens (ZO)-1, which are typically expressed in corneal epithelial cells, was also determined. Cytokeratin 15 (KRT15) and the ATP-binding cassette transporter G2 (ABCG2) are both markers of LESCs.[33] Both HCE cells and outgrowths of limbal explants were harvested at confluence. Total RNA was isolated using the RNeasy® Mini Kit (Quiagen, Valencia, CA, USA*)* according to specifications of the manufacturer.[34, 35] The RNA concentration was measured by a fluorometric method using the Quant-iT RNA assay and treated with RNase-Free DNase Set (Qiagen).

Total RNA (1 μg) was retrotranscribed to cDNA using SuperScript® Vilo™ cDNA Synthesis Kit (Invitrogen-Gibco). The RNA was mixed with 5X Vilo Reaction Mix and 10X Superscript Enzyme Mix (7:1:2) and thermocycled at 25ºC for 10 min, at 42ºC for 120 min, and finally at 85ºC for 5 min. PCR amplifications using specific probes (Table 1) were performed in a PCR 7500 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to specifications of the manufacturer.

All experiments were performed in duplicate. A non-template negative control was included in all experiments to evaluate DNA and RT-PCR contamination of the reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for each reaction. The comparative cycle threshold (Ct) method[36] (Applied Biosystems User Bulletin, No.2; P/N 4303859) was used for analyzing the results. For HCE cells, four independent experiments were performed. For the limbal epithelial cells cultured on different substrata, 5 - 7 experiments from different donors were carried out.

Immunofluorescence staining

Cultured cells were harvested by incubation for 30 min at 37ºC with Versene. A total of 50,000 cells in 250 μl of DMEM-F12 were pelleted by cytocentrifugation for 10 min at 800 rpm with low

acceleration. Cells were deposited onto a 28 mm^2 area of a poly-L-lysine–treated glass slide and fixed with cold methanol at -20ºC for 10 min.

For immunofluorescence staining, the cells were incubated for 1 h at room temperature with blocking buffer of 5% donkey serum in PBS. The cells were then incubated with primary antibody (Table 2) in a humidified chamber at 4ºC overnight. Afterwards, they were rinsed with PBS and incubated with the secondary antibody (Table 2) in the dark at 37ºC for 1 h. Nuclei were counterstained with propidium iodide and analyzed under fluorescent microscope (Leica DMI 6000 B, Leica).

Positively and negatively stained cells in 5 random fields of 4 independent HCE cultures and 4 independent explant cultures were counted. The percentage of positive cells was calculated for each field, and the mean percentage of positive cells for each marker was determined.

Statistical analysis

Statistical significance was determined by one-way factorial ANOVA. Comparison between two groups was made using Student's t-test. All values were expressed as means ± SEMs. P-values ≤ 0.05 were considered statistically significant.

Results

Physical-chemical characterization

The polymeric membranes obtained by casting in the 10 cm molds had a homogeneous yellowish appearance. The color intensity increased as the content of chitosan increased. The chemical structure of the different membranes was analyzed by FTIR (Fig.1A). FTIR spectrum of raw chitosan showed the typical amide I (C=O) at 1660 cm^{-1}, amide II (N-H bending) at 1590 cm^{-1}, and amide III at 1316 cm^{-1}. FTIR spectrum of raw gelatin showed the amide I band al 1630 cm^{-1}, amide II (N-H bending and C-N stretching) at 1540 cm^{-1}, and amide III at 1240 cm^{-1}. Chitosan-gelatin crosslinked membranes presented bands of both compounds whose intensities depended on the membrane composition.

The thermal stability and thermal degradation processes of polymeric materials are related to the microstructure, especially the intra- and inter-molecular interactions and between the polymeric chains. Thermal degradation of CH-G (80:20) and (50:50) membranes (Fig.1B) underwent in only one step and for these samples, the maximum degradation rate of chitosan (284ºC) moved to higher temperatures as the content of gelatin increased in the polymeric membranes, reaching 296ºC for CH-G 50:50. This fact

reflects a good interconnection of both types of polymeric chains in the membrane, which could be due to the crosslinking reaction but also to entanglements formation between the different macromolecular chains that can be stabilized by hydrogen bonds. However, for the CH-G 20:80 sample, two maxima were present; indicating that interconnection between gelatin and chitosan macromolecules is not so strong when gelatin is in such a proportion.

Swelling experiments demonstrated pH sensitivity associated with the presence of ionizable groups (Fig.2A-C). The primary amino groups in chitosan molecules are ionized at acidic pH, and the ionic character of the chitosan at pH 2 increased the hydrophilicity of the membranes, increasing the swelling. Gelatin membranes were also sensitive to pH. However when compared to chitosan, pH had a minimal effect on the swelling of these membranes. In composite membranes, higher gelatin content was associated with greater swelling at pH 10 and 7.4. However, the opposite trend occurred at pH 2. Complete degradation of pure gelatin membranes took place after 1, 2, and 4 days at pH 2, 7.4, and 10, respectively (data not shown). Swelling experiments were carried out during 14 days or until the tested sample disintegrated in the corresponding media. Gelatin and CH-G 20:80 lost stability in acidic buffered solution after 24 hours. Gelatin lost stability at pH 7.4 or 10 after 2 or 4 days respectively. In CH-G (20:80) membranes, swelling at pH 7.4 peaked at 90 min, reaching 450%. However, by 150 min, the swelling decreased to 344%. There was no weight loss in any other membrane after 2 weeks at any pH, so chitosan incorporation increased membrane stability at all pHs.

HCE cell viability and proliferation

Prior to the cellular studies, the membranes composed of CH-G 80:20 were discarded because they were hard and brittle, indicating that this mixture was not appropriate for the future transplantation to the ocular surface. Further, membranes composed of gelatin alone were not suitable due to degradation after few days in culture medium. In contrast, membranes composed of CH-G 20:80, CH-G 50:50, and chitosan alone were easy to handle in the swollen state.

HCE cells were seeded onto membranes composed of chitosan alone (CH-G 100:0), and mixtures of CH-G 50:50 and 20:80. Within 6 h, most of the cells adhered to all substrata, including the TCP control, and all had similar viabilities of >90% ($p>0.05$, Table 3) for the 8-day study period.

HCE cell proliferation on each substratum was assessed by AB testing at 1, 2, 4, and 8 days in culture. The relative cell density for cells seeded onto CH-G 50:50 and CH-G 20:80 was similar after 8 days in culture (Fig.3). At the same time, the density of cells grown on CH-G 100:0 was lower than that on the other CH-G mixtures although the differences were not significant (Fig.3). Because of these trends, we excluded CH-G 100:0 from further experiments.

Specific marker expression in HCE cells cultured on chitosan-gelatin biopolymers

After 8 days of culture, the relative expression levels of KRT3 and KRT12 mRNAs (Fig.4A) were not significantly different in cells grown on CH-G 20:80 or CH-G 50:50 when compared with the control substratum TCP. There were also no differences for cells grown on CH-G 20:80 or CH-G 50:50 compared to cells grown on TCP in the relative expression of mRNAs for E-cadherin, desmoplakin, and ZO-1, proteins found in intercellular junctions (Fig.4B). Despite the absence of significant differences, the expression of marker protein mRNAs in cells grown on CH-G 20:80 was more similar to cells grown on TCP than were cells grown on CH-G 50:50. For this reason, we chose CH-G 20:80 for further studies as the most suitable substratum.

The protein expression level of K3 and K12 was analyzed by immunofluorescence staining in HCE cells grown on CH-G 20:80 and TCP (Fig.4C, D). After 8 days in culture, the percentage of cells positive for K3 and K12 was always above 90% with no significant differences between the tested substrata.

These results indicated that CH-G 20:80 polymer supported cell viability and proliferation and maintained the expression levels of differentiated corneal epithelial cell markers compared with TCP. This suggested that CH-G 20:80 could be a good carrier substratum for LESCs.

Limbal epithelial cell isolation and expansion on CH-G 20:80

Human limbal epithelial cells were grown from 44 limbal superior and inferior explants obtained from 8 different cadaveric donor corneoscleral rings. For explants seeded on CH-G 20:80, 54.0 ± 0.1% grew and reached confluence, similar to the 58.0 ± 0.1% on TCP. Growth of cells from the explanted limbal tissue was first detected on day 12.3 ± 1.1 and 12.0 ± 1.2 of culture on CH-G 20:80 and TCP, respectively. Cellular confluence was achieved 23.3 ± 1.1 days after initiating explant cultures on CH-G 20:80 and 22.8 ± 1.9 days on TCP. There were no significant differences between cultures grown on CH-G 20:80 and TCP in the percent of cultures reaching confluence, the detection of initial growth, or the

time to reach confluence. These results suggest that the substratum CH-G 20:80 did not affect the growth of limbal epithelial cells compared to explants grown on TCP.

Explant evolution and cell morphology were monitored by light and phase contrast microscopy (Fig.5A). For both substrata, the outgrowth developed as a monolayer of cells. The cells were morphologically heterogeneous, some being small in size and some larger, but most of them presented the polygonal structure typical of epithelial cells.

Specific marker expression in primary limbal epithelial cell cultures

The relative gene expression of KRT3, 12, 15, and of the transporter ABCG2 was analyzed in limbal epithelial cells grown on CH-G 20:80 and TCP. KRT3 and KRT12 mRNAs, both of which are differentiated corneal epithelial cell markers, were expressed in cells grown on CH-G 20:80 at similar levels compared cells grown on TCP (Fig.5B). In contrast, expression of the specific LESC marker KRT15 was significantly higher ($p<0.05$) in cells grown on CH-G 20:80 than in those grown on TCP (Fig.5B). Although the LESC marker ABCG2 was highly expressed in cells grown on CH-G 20:80, mRNA expression levels were slightly but significantly lower ($p<0.05$) than in cells grown on TCP (Fig.5B).

The relative expression level among different LESC markers in cells cultured on CH-G 20:80 were compared using KRT3 as a calibrator (Fig.5C). Expression of the LESC mRNA marker KRT15 was significantly higher ($p<0.05$) than that of KRT3 and KRT12, both of which are considered to be markers of corneal epithelial differentiation. The expression of the stem cell marker ABCG2 was significantly lower ($p<0.05$) than KRT15, but not different from KRT3 or KRT12 (Fig.5C).

By immunofluorescence microscopy, significantly fewer cells cultured on TCP expressed K3 and K12 proteins (76% and 86% respectively) than those on CH-G 20:80 ($p<0.05$, Fig.5D). The specific limbal epithelial cytokeratin K15 was expressed in about 50% of cells on both substrata (Fig.5D). Furthermore, approximately 90% of cells grown on TCP and CH-G 20:80 expressed the LESC-specific transporter protein ABCG2 (Fig.5D).

DISCUSSION

One method for restoring the integrity of the corneal epithelium damaged by trauma or disease is to provide a source of stem cells that then produce cells that differentiate and resurface the cornea. Furthermore, a carrier substratum is necessary for the *in vitro* expansion of epithelial cells and the

subsequent transplantation onto the ocular surface. Thus, the aim of our study was to evaluate different biopolymers based on chitosan and gelatin as potential carrier substrata for human limbal epithelial cells.

HAM is a commonly used biological substratum for LESCs.[9, 37] It is good for ocular surface reconstruction because it is non-immunogenic, anti-inflammatory, and promotes epithelial cell adhesion and proliferation.[38-40] However, as a natural product, the biological variations in HAM between donors cannot be controlled, and it is not optically clear. Even more importantly, the content of EGF varies among membranes and at different sites within the same membrane. Thus HAM is not sufficiently standardized fot use as a tissue construct for *in vitro* expansion of cells.[12-14] Additionally, the limited availability of donor tissue, and the potential transmission of diseases have prompted the exploration for alternative solutions.[41]

Most of the published studies utilized non-human cells, e.g., mouse 3T3 fibroblasts, as a feeder layer and animal-derived products in the culture media.[11, 42] The presence of non-human animal products in cell cultures destined for future human transplantation holds the potential of transmitting source animal diseases to humans.[37] Thus, to better standardize the cell cultures and minimize any risk for future patients, we used semisynthetic polymers as carrier substrata. Additionally, we cultured the limbal epithelial cells directly on them, without any other supporting cell lineage or products derived from non-human animal sources.

Chitosan, with structural characteristics similar to glycosaminoglycans, has been used to mimic principal components of the cornea and other tissues, resulting efficient in the regeneration processes of different tissues and wound healing.[43, 44] It has numerous and important biological properties[45] such as low immunogenicity, antibacterial activity, mucoadhesivity, low cytotoxicity, biodegradability, and wound healing activity.[46] Furthermore, chitosan enhances cell adhesion and proliferation, as documented with osteoblasts, fibroblasts, keratinocytes, and endothelial cells.[44-46] Chitosan and gelatin have been used together for reconstruction and repair[23] in bone and cartilage regeneration[47] and as a carrier substratum for epithelial cells in skin regeneration.[48] In the eye, chitosan and gelatin, combined with chondroitin sulfate, were used to develop an artificial cornea that induced new corneal tissue formation in rabbits, improving the adhesion and growth of rabbit corneal epithelial cells.[49] Chitosan is an effective carrier substratum for conjunctival epithelial cells, and its mechanical and biological properties are improved by the addition of gelatin.[46] For these reasons, we included gelatin in the chitosan–based membranes to improve chemical, physical, and biological properties.

In our study, we constructed membranes based on different proportions of chitosan and gelatin that were stabilized by crosslinking and by hydrogen bond interactions. Membranes richer in gelatin were more rapidly biodegradable. This phenomenon is not a drawback for a substratum that is to be used for tissue regeneration. On the contrary, it is desirable that the substratum or scaffold is degraded as the cells proliferate and produce new tissue. Membranes with a higher content of chitosan were more stable but also more rigid and brittle. Membranes based on CH and G were more efficiently crosslinked when CH is the main component of the formulation, as more amino groups are included in the structure. Moreover, G is more hydrophilic than CH and therefore absorbs higher quantities of aqueous media. Therefore, those crosslinked membranes based on CH as main component are more brittle and less hydrophilic.

This was the reason for the exclusion of the membranes based on CH-G 80:20, since they were too stiff to be used as a transplantable substratum.

Currently, the availability of LESCs is limited, and they are difficult to obtain for primary cultures. Therefore we conducted the studies of biocompatibility (cell viability and proliferation) and specific marker expression with the HCE cell line because it is abundant and relatively inexpensive. Additionally, these cells are similar to the ones that the *in vivo* offspring of LESCs become under normal circumstances. Based on these results, we then chose the most promising surface for the subsequent limbal epithelial cell expansion. None of the polymers were cytotoxic, and HCE cell viability was similar among the different substrata compositions and the TCP.

Gelatin had a positive effect on cell growth. Proliferation increased when cells were cultured on gelatin-containing chitosan-based substrata. Zhu et al. observed that this effect was critical for conjunctival cell proliferation for which the optimal gelatin component was around 50%.[41] However in our study, after 8 days in culture, there were no differences in cell proliferation between CH-G 50:50 and CH-G 20:80.

Because the ultimate application of these polymers will be as carriers for LESCs, it was important to determine if the experimental substrata induced changes in gene and protein expression. Expression of KRT3 and KRT12 are typical for HCE and normal corneal epithelial cells.[30-32] Furthermore, corneal epithelial cells are strongly attached to one another by desmosomes and tight junctions,[50] and they express adhesion proteins E-cadherin, desmoplakin, and ZO-1.[50-52] Therefore we used these biomarkers to detect any changes in mRNA and protein expression induced by the different substrata. For HCE cells, expression of the specific cytokeratin and adhesion protein mRNA levels on CH-G 20:80 was

closer to that of the control TCP than were either CH-G 100:0 or CH-G 50:50. For that reason, in addition to the suitable viability and growth rates, we selected the CH-G 20:80 polymer as the best candidate for the LESC expansion.

The limbal specific undifferentiated stem cell markers K15 and ABCG2 and the specific differentiated corneal epithelial cytokeratins K3 and K12 were expressed in the limbal cells cultured up to confluence on CH-G 20:80. This indicated a heterogeneous population of corneo-limbal cells, as is present in the native limbal niche.[53, 54] Real time RT-PCR showed that KRT15 was expressed at greater levels in explant outgrowths on CH-G 20:80 than on TCP. In limbal cells grown on CH-G 20:80, KRT15 was also present at higher levels than KRT3, KRT12, and ABCG2. These expression levels suggest that this polymer could serve as a carrier for a stem cell-containing population of limbal epithelial cells, and also for the subsequently differentiated corneal epithelial cells.

As observed by immunofluorescence microscopy, the percentage of cells expressing LESC markers K15 and ABCG2 was similar for cells grown on CH-G 20:80 and TCP. This suggests that LESCs maintain phenotype when cultured on this biopolymer. The percentage of cells that expressed K3 and K12 by immunofluorescence microscopy was higher for explant outgrowths on CH-G 20:80, suggesting that the final state of these LESCs expanded on this polymer included more differentiated corneal epithelial cells.

Less than 10% of basal epithelial cell population located at the limbal niche are considered to be stem cells.[55, 56] For that reason, K15 was present in a lower percentage of cells compared to K3 and K12 obtained from limbal explants, but the mRNA expression level measured by real time RT-PCR was higher in those cells. These results indicate that a heterogeneous population of LESCs and differentiated corneal epithelial cells were obtained from limbal explants grown on CH-G 20:80. The significantly higher expression level of K15 and the expression of ABCG2 demonstrate that this substratum could be appropriate for the *in vitro* expansion of a stem cell-containing population of limbal epithelial cells for subsequent transplantation.

In summary, we have demonstrated the use of a biopolymer substratum made of chitosan crosslinked with gelatin for the expansion and growth of a stem cell-containing population of limbal epithelial cells. For cases of LESC deficiency, this new substratum is a candidate for expanding LESCs for transplantation onto damaged ocular surface.

REFERENCES

[1] Ahmad S, Figueiredo F, Lako M (2006) Regen Med 1:29

[2] Kenyon KR, Tseng SC (1989) Ophthalmology 96:709

[3] Garg P, Krishna PV, Stratis AK et al (2005) Eye (Lond) 19:1106

[4] Pellegrini G, Traverso CE, Franzi AT et al (1997) Lancet 349:990

[5] Koizumi N, Inatomi T, Suzuki T et al (2001) Ophthalmology 108:1569

[6] Miri A, Al-Deiri B, Dua HS (2010) Ophthalmology 117:1207

[7] Rama P, Bonini S, Lambiase A et al (2001) Transplantation 72:1478

[8] Sangwan VS, Matalia HP, Vemuganti GK et al (2006) Indian J Ophthalmol 54:29

[9] Shortt AJ, Secker GA, Rajan MS et al (2008) Ophthalmology 115:1989

[10] Deshpande P, Notara M, Bullett N et al (2009) Tissue Eng Part A 15:2889

[11] Levis HJ, Brown RA, Daniels JT (2010) Biomaterials 31:7726

[12] Gicquel JJ, Dua HS, Brodie A et al (2009) Tissue Eng Part A 15:1919

[13] Koizumi N, Cooper LJ, Fullwood NJ et al (2002) Invest Ophthalmol Vis Sci 43:2114

[14] Pratoomsoot C, Tanioka H, Hori K et al (2008) Biomaterials 29:272

[15] Langer R, Vacanti JP (1993) Science 260:920

[16] Fisher OZ, Khademhosseini A, Langer R et al (2010) Acc Chem Res 43:419

[17] Ang LP, Cheng ZY, Beuerman RW et al (2006) Invest Ophthalmol Vis Sci 47:105

[18] Martinez-Osorio H, Juarez-Campo M, Diebold Y et al (2009) Curr Eye Res 34:48

[19] Minami Y, Sugihara H, Oono S (1993) Invest Ophthalmol Vis Sci 34:2316

[20] Niiya A, Matsumoto Y, Ishibashi T et al (1997) Graefes Arch Clin Exp Ophthalmol 235:32

[21] Rodriguez-Cabello JC, Prieto S, Reguera J et al (2007) J Biomater Sci Polym Ed 18:269

[22] Dravida S, Gaddipati S, Griffith M et al (2008) J Tissue Eng Regen Med 2:263

[23] Khor E, Lim LY (2003) Biomaterials 24:2339

[24] Manjubala I, Ponomarev I, Wilke I et al (2008) J Biomed Mater Res B Appl Biomater 84:7

[25] Pulieri E, Chiono V, Ciardelli G et al (2008) J Biomed Mater Res A 86:311

[26] Mariappan I, Maddileti S, Savy S et al (2010) Nat Protoc 5:1470

[27] Mao JS, Zhao LG, Yin YJ et al (2003) Biomaterials 24:1067

[28] Araki-Sasaki K, Ohashi Y, Sasabe T et al (1995) Invest Ophthalmol Vis Sci 36:614

[29] Takahashi N, Chikama T, Yanai R et al (2009) Jpn J Ophthalmol 53:199

[30] Kurpakus MA, Maniaci MT, Esco M (1994) Curr Eye Res 13:805

[31] Rodrigues MM, Krachmer J, Rajagopalan S et al (1987) Cornea 6:190

[32] Schermer A, Galvin S, Sun TT (1986) J Cell Biol 103:49

[33] Yoshida S, Shimmura S, Kawakita T et al (2006) Invest Ophthalmol Vis Sci 47:4780

[34] Corrales RM, Calonge M, Herreras JM et al (2003) Curr Eye Res 27:323

[35] Luo L, Li DQ, Doshi A et al (2004) Invest Ophthalmol Vis Sci 45:4293

[36] Livak KJ, Schmittgen TD (2001) Methods 25:402

[37] Kolli S, Ahmad S, Lako M et al (2010) Stem Cells 28:597

[38] Anderson DF, Ellies P, Pires RT et al (2001) Br J Ophthalmol 85:567

[39] Koizumi N, Fullwood NJ, Bairaktaris G et al (2000) Invest Ophthalmol Vis Sci 41:2506

[40] Sangwan VS, Burman S, Tejwani S et al (2007) Indian J Ophthalmol 55:251

[41] Zhu X, Beuerman RW, Chan-Park MB et al (2006) Ann Acad Med Singapore 35:210

[42] Balasubramanian S, Jasty S, Sitalakshmi G et al (2008) Indian J Med Res 128:616

[43] Chandy T, Sharma CP (1990) Biomater Artif Cells Artif Organs 18:1

[44] Suh JK, Matthew HW (2000) Biomaterials 21:2589

[45] Muzzarelli R, Baldassarre V, Conti F et al (1988) Biomaterials 9:247

[46] Gao X, Liu W, Han B et al (2008) Sheng Wu Gong Cheng Xue Bao 24:1381

[47] Bhat S, Tripathi A, Kumar A (2011) J R Soc Interface 8:540

[48] Liu H, Mao J, Yao K et al (2004) J Biomater Sci Polym Ed 15:25

[49] Huang YX, Li QH (2007) Biomed Mater 2:S121

[50] Gonzalez-Andrades M, Garzon I, Gascon MI et al (2009) J Tissue Eng Regen Med 3:442

[51] Chen Z, de Paiva CS, Luo L et al (2004) Stem Cells 22:355

[52] Higa K, Shimmura S, Miyashita H et al (2009) Invest Ophthalmol Vis Sci 50:4640

[53] Li W, Hayashida Y, Chen YT et al (2007) Cell Res 17:26

[54] Schlotzer-Schrehardt U, Kruse FE (2005) Exp Eye Res 81:247

[55] Lavker RM, Dong G, Cheng SZ et al (1991) Invest Ophthalmol Vis Sci 32:1864

[56] Merjava S, Brejchova K, Vernon A et al (2011) Invest Ophthalmol Vis Sci 52:787

ACKNOWLEDGMENTS

This work has been supported by the Instituto de Salud Carlos III (CIBER-BBN CB06/01/003), Spain. Centro en Red de Medicina Regenerativa y Terapia Celular, Castilla y León, Spain. Junta de Castilla y León, Spain: SAN673/VA/28/08, SAN126/VA11/09.

CIBER-BBN is an initiative funded by the VI National R&D&i Plan *2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions* and financed by the Instituto de Salud Carlos III with assistance from the *European Regional Development Fund*.

S. Galindo and M. López-Paniagua were supported by scholarships co-financed by the Castilla y León Government and the European Social Fond.

We thank M. F. de la Paz, MD, (Barraquer Eye Bank of Barcelona, Spain) and J. Iglesias, PhD, (Tissue Establishment, San Francisco Clinic Fundation, Leon, Spain) for providing human corneoscleral tissues. We also thank J. M. Herreras, MD, for his clinical advice, V. Sáez (IOBA laboratory technician) for her initial technical assistance and I. Fernández (IOBA, University of Valladolid, Spain) for statistical assistance

We thank S. Gonzalo for her technical support in the polymer characterization experiments.

And we thank B. Bromberg (Certified Editor in Life Science of Xenofile Editing, www.xenofileediting.com) for his assistance in the final editing and preparation of this manuscript.

Presented in part as an abstract at the 80th Annual meeting of the Association for Research in Vision and Ophtalmology (ARVO), May 2009, Fort Lauderdale, FL, USA (IOVS 2009, ARVO-E-Abstract 6288); at the 22nd European Conference on Biomaterials (ESB2009), September 2009, Lausanne (Switzerland), poster 715 0102; and at the Biofuture 2011: Young European Biomaterial Scientists Designing a View for the Future, November 2011, Gante (Belgium)

No competing financial interests exist.

Figure legends

Fig. 1 (A) FTIR spectra of the synthesized membranes. The spectra showed the typical amide I (C=O) at 1660 cm^{-1}, amide II (N-H bending) at 1590 cm^{-1}, and amide III at 1260 cm^{-1} bands for chitosan. For gelatin, the amide I band was at 1630 cm^{-1}, amide II (N-H bending and C-N stretching) was at 1540 cm^{-1}, and amide III was at 1240 cm^{-1}. **(B)** The first derivative of the TGA thermograms for the chitosan-gelatin membranes indicates the degradation rate of the synthesized membranes as a function of temperature.

Fig. 2 Swelling as a function of chitosan and gelatin composition. **(A)** pH 2, **(B)** pH 7.4, **(C)** pH 10.

Fig. 3 Human corneal epithelial (HCE) cell line proliferation. Relative cell density of cells cultured for 8 days on chitosan-gelatin polymers at different CH-G ratios: CH-G 100:0, CH-G 50:50, CH-G 20:80, and tissue culture plastic (TCP) as a control substratum. Data obtained from 4 independent experiments.

Fig. 4 Relative mRNA expression levels of HCE cell cytokeratins **(A)** (KRT3 and KRT12) and **(B)** adhesion proteins (E-cadherin (E-cad), desmoplakin (Dsp), and zonula occludens 1 (ZO-1)) after 8 days of culture on TCP, CH-G 50:50, and CH-G 20:80 ($p>0.05$, $n=4$). **(C)** Immunofluorescence staining of cytokeratins K2 and K12 in HCE cells after 8 days of culture on tissue culture plastic (TCP) and CH-G 20:80. Red, nuclear counterstain with propidium iodide; magnification 40X. Micrographs are representative of 4 independent experiments. **(D)** Percentage of K3 and K12 positive cells on TCP and CH-G 20:80 substrata.

Fig. 5 Outgrowths from human cadaveric corneoscleral limbal explants. **(A, left)** Explant on CH-G 20:80 biopolymer. (**A, middle)** Explants growth first detection (12.3 ± 1.1 days) (n = 44 explants from 8 different donors); magnification 20X. **(A, right)** Cell confluence (23.3 ± 1.1 days) (n = 24 explants from 8 different donors); magnification 20X. **(B)** Relative mRNA expression levels of limbal (KRT15 and ABCG2) and corneal (KRT3 and KRT12) epithelial cells markers using the expression on TCP as a calibrator and **(C)** using KRT3 expression on CH-G 20:80 as calibrator (*$p<0.05$). **(D)** Immunofluoresce staining (green) for K15, ABCG2, K3 and K12. Red, nuclear counterstain with propidium iodide; magnification 40X. Micrographs are representative of 4 independent experiments.

Fig 1

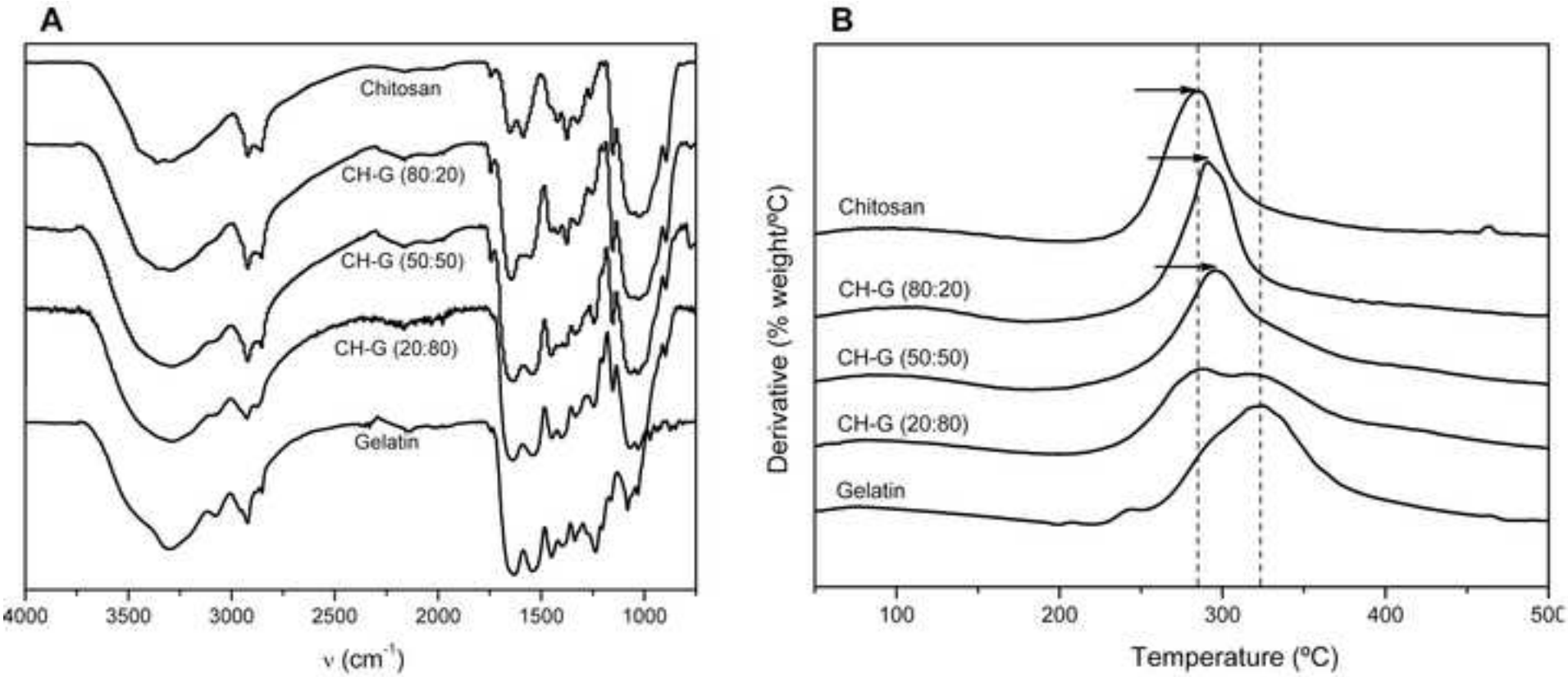
A
Chitosan
CH-G (80:20)
CH-G (50:50)
CH-G (20:80)
Gelatin
4000
3500
3000
2500
2000
1500
1000
ν (cm-1)
B
Derivative (% weight/ºC)
Chitosan
CH-G (80:20)
CH-G (50:50)
CH-G (20:80)
Gelatin
100
200
300
400
500
Temperature (ºC)

Fig 2

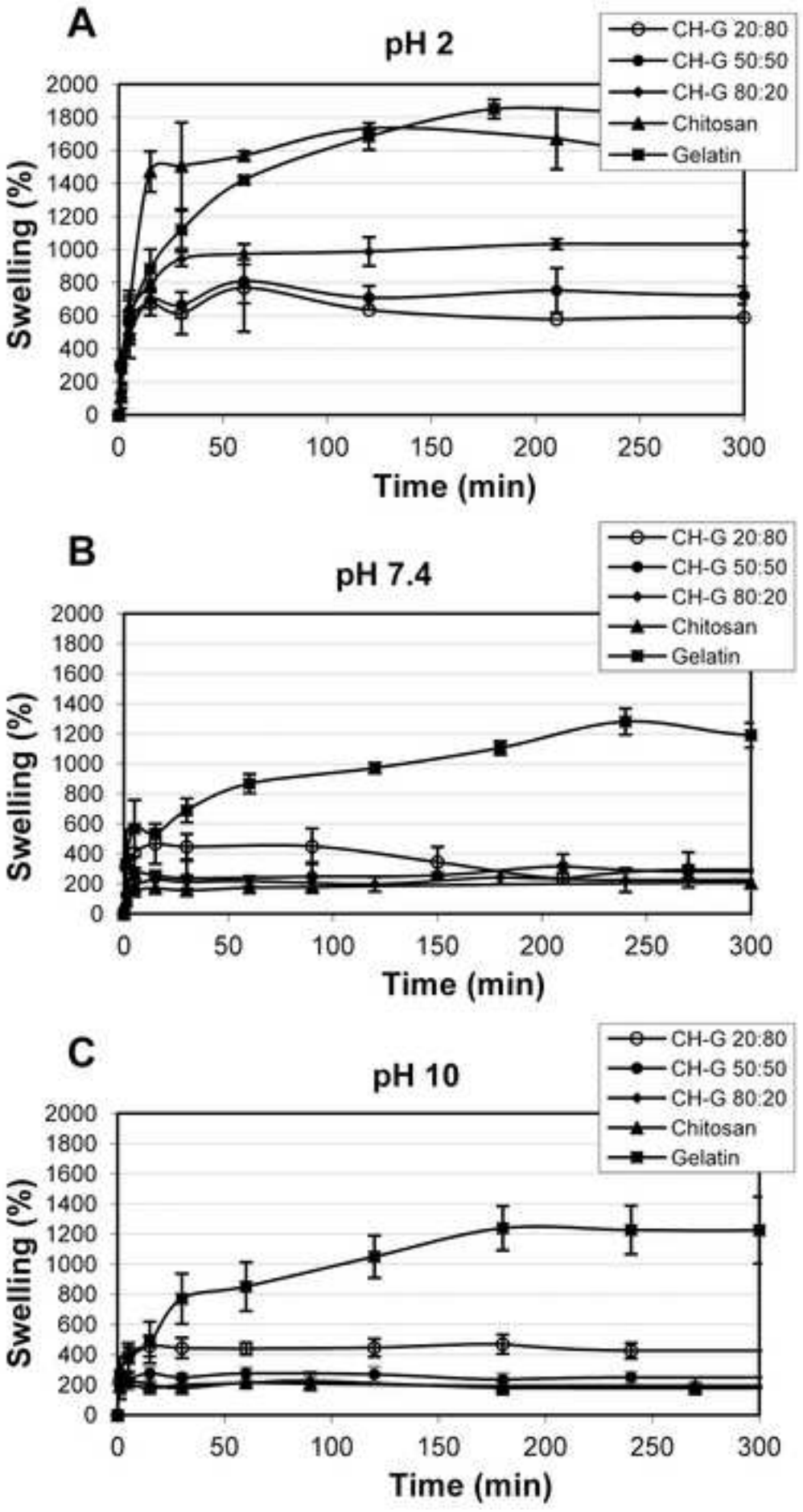
A
pH 2
B
pH 7.4
C
pH 10
CH-G 20:80
CH-G 50:50
CH-G 80:20
Chitosan
Gelatin
Swelling (%)
Time (min)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
0
50
100
150
200
250
300

Fig 3

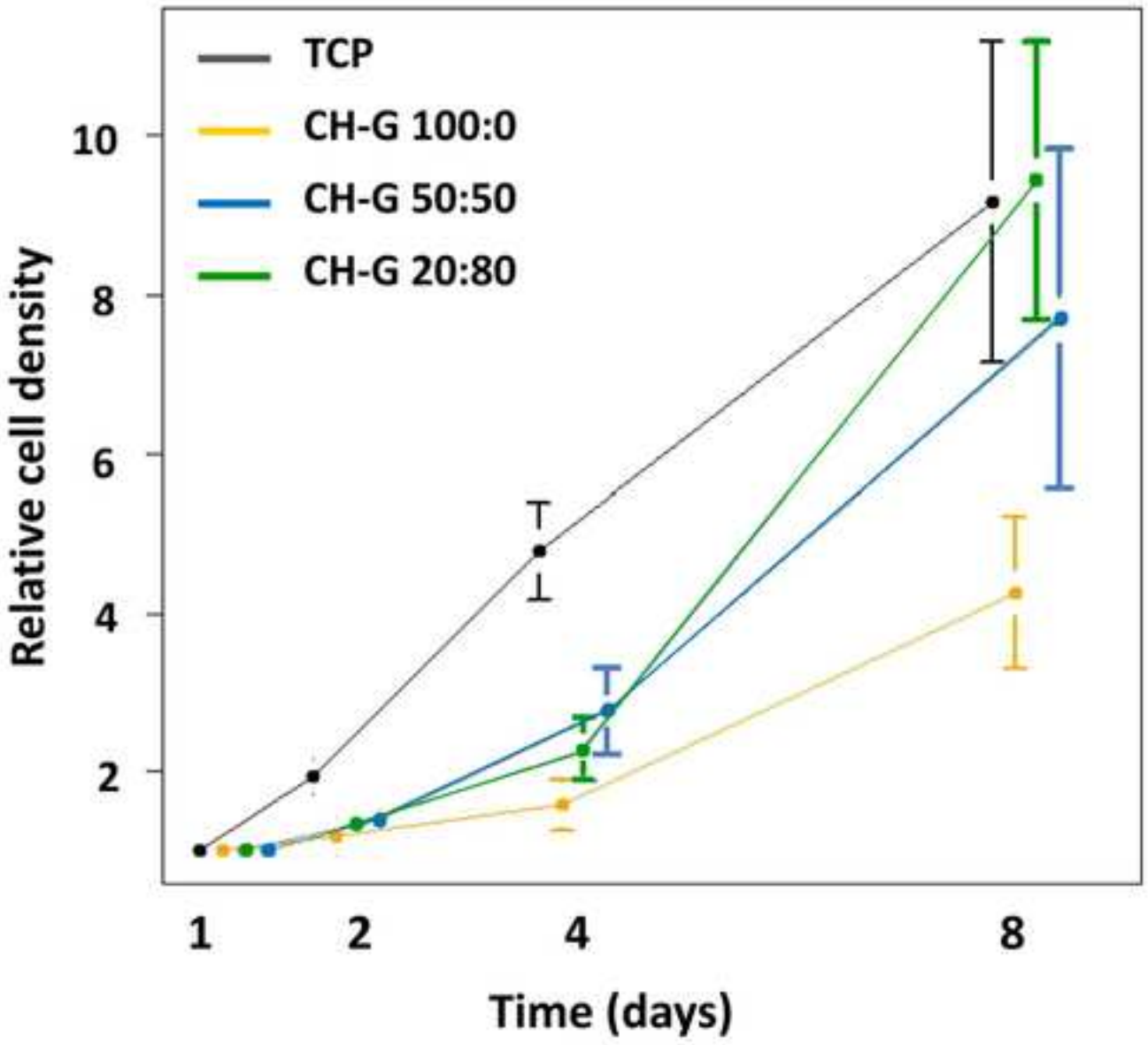
TCP
CH-G 100:0
CH-G 50:50
CH-G 20:80
Relative cell density
10
8
6
4
2
1
2
4
8
Time (days)

Fig 4

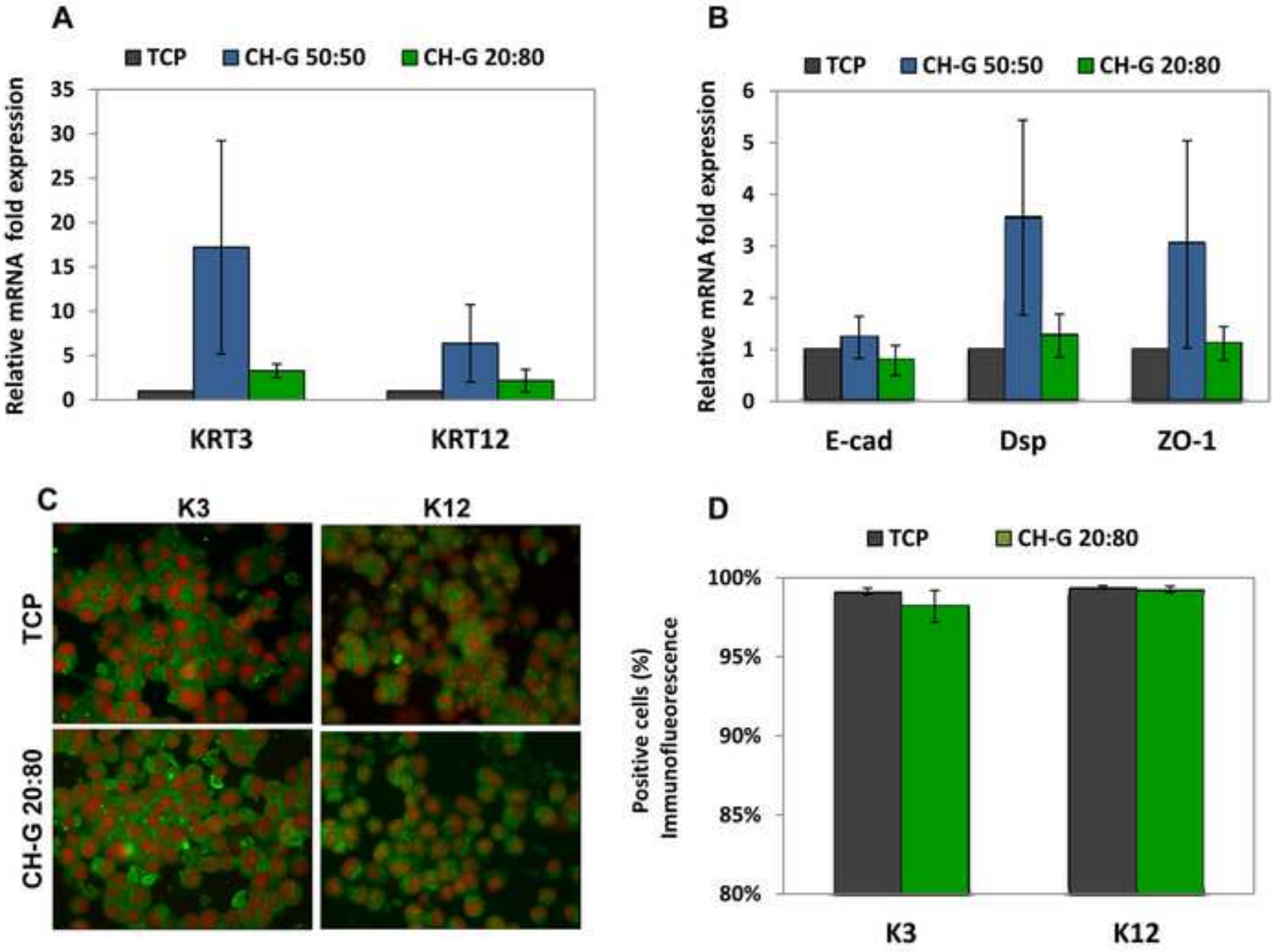
A
TCP
CH-G 50:50
CH-G 20:80
Relative mRNA fold expression
35
30
25
20
15
10
5
0
KRT3
KRT12
B
TCP
CH-G 50:50
CH-G 20:80
Relative mRNA fold expression
6
5
4
3
2
1
0
E-cad
Dsp
ZO-1
C
K3
K12
TCP
CH-G 20:80
D
TCP
CH-G 20:80
Positive cells (%) Immunofluoresecence
100%
95%
90%
85%
80%
K3
K12

Fig 5

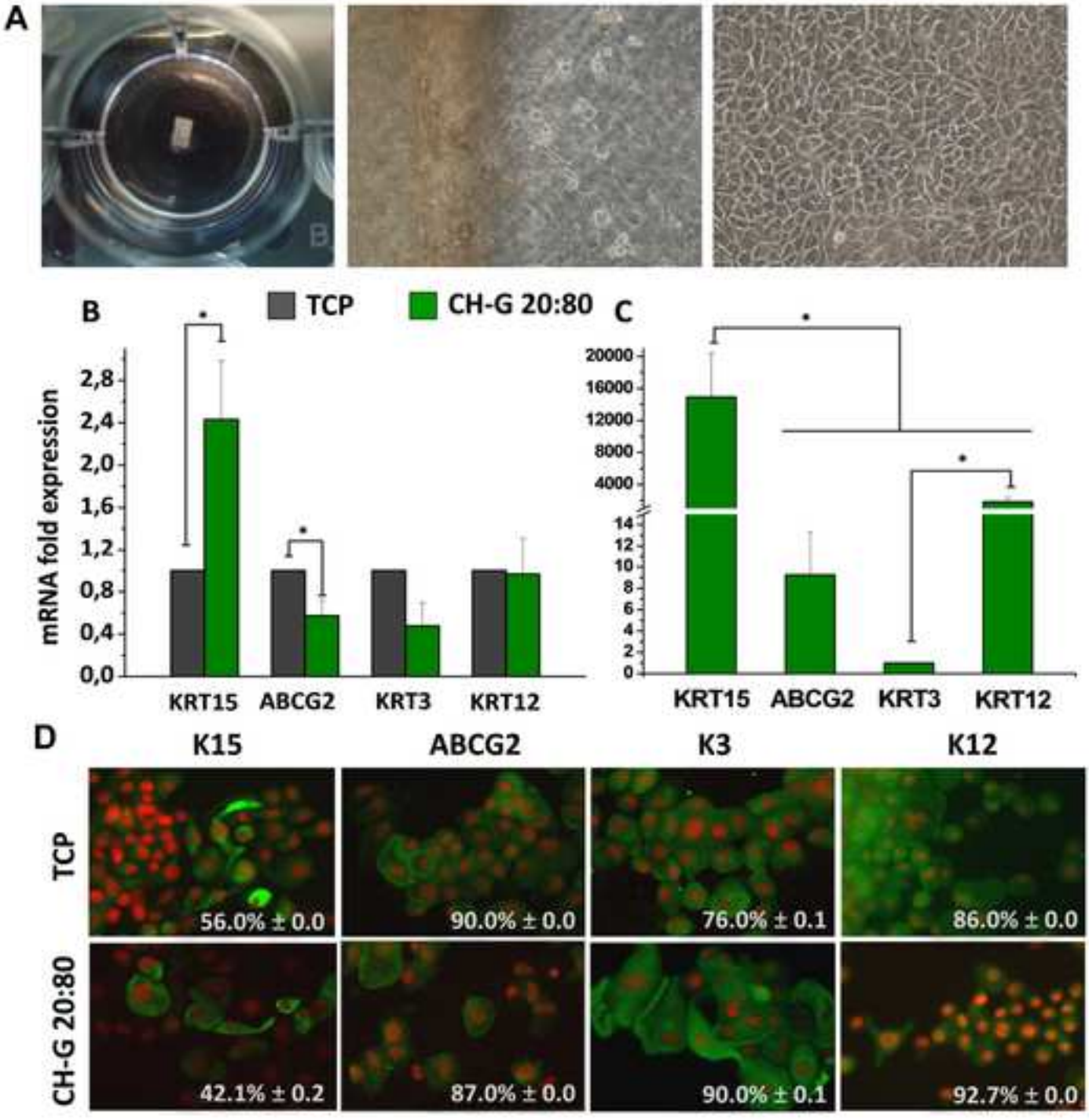
A
B
TCP
CH-G 20:80
mRNA fold expression
2,8
2,4
2,0
1,6
1,2
0,8
0,4
0,0
KRT15
ABCG2
KRT3
KRT12
C
20000
16000
12000
8000
4000
14
12
10
8
6
4
2
0
KRT15
ABCG2
KRT3
KRT12
D
K15
ABCG2
K3
K12
TCP
56.0% ± 0.0
90.0% ± 0.0
76.0% ± 0.1
86.0% ± 0.0
CH-G 20:80
42.1% ± 0.2
87.0% ± 0.0
90.0% ± 0.1
92.7% ± 0.0

Table 1. Taqman probes used for real time PCR analysis

Gene name	Symbol	Identification number of Applied Biosystems
Glyceraldehide-3-phosphate dehydrogenase	GAPDH	4352934E
Desmoplakin (DSP)	DSP	Hs00189422_m1
E-cadherin (CDH1)	E-cad	Hs01023894_mi
Zonula occludens 1 (TJP1)	ZO-1	Hs00268480_m1
ATP-binding cassette transporter G2	ABCG2	Hs00184979_m1
Cytokeratin 3 (KRT3)	KRT3	Hs00365080_m1
Cytokeratin 12 (KRT12)	KRT12	Hs00165015_m1
Cytokeratin 15 (KRT15)	KRT15	Hs00267035_m1

Table 2

Table 2. Primary and secondary antibodies used for immunofluorescence staining

Antibodies	Category (monoclonal)	Catalog No.	Source	Working dilution
PRIMARY				
Cytokeratin AE5 (K3)	Mouse	69143	Mp Biomedical (Illkirch, France)	1:50
Cytokeratin 12 (K12)	Rabbit	25722	Sta Cruz (Heidelberg, Germany)	1:50
Cytokeratin 15 (K15)	Mouse	CBL272	Chemicon/Millipore (Billerica, MA, USA)	1:50
ABCG2	Mouse	MAB4146	Chemicon/Millipore (Billerica, MA, USA)	1:20
SECONDARY				
Alexa fluor 488 donkey anti-mouse IgG			Invitrogen (Inchinnan, UK)	1:200
Alexa fluor 488 donkey anti-rabbit IgG			Invitrogen (Inchinnan, UK)	1:300

Table 3. Cell viability of the human corneal epithelial cell line (HCE).

Substrates	Cell viability (%)				
	6h	24h	48h	4days	8days
TCP	99.6 ± 0.0	98.0 ± 0.0	99.0 ± 0.0	97.3 ± 0.0	99.0 ± 0.0
CH-G 100:0	98.0 ± 0.0	96.5 ± 0.0	100.0 ± 0.0	99.0 ± 0.0	98.0 ± 0.0
CH-G 50:50	98.0 ± 0.0	96.3 ± 0.0	99.3 ± 0.0	97.0 ± 0.0	97.0 ± 0.0
CH-G 20:80	98.3 ± 0.0	97.0 ± 2.0	96.5 ± 0.0	96.0 ± 0.0	91.0 ± 0.0

TCP, tissue culture plastic; CH, chitosa; G, gelatin. Mean ± SEM based upon cell counts of 5 random fields of 4 independent experiments