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Short communication

Ulvan and ulvan/chitosan polyelectrolyte nanofibrous membranes as a potential substrate material for the cultivation of osteoblasts

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ABSTRACT

A new generation of biomaterials composed of the natural polysaccharides, ulvans extracted from the green seaweed *Ulva rigida* and chitosan have been investigated. Ulvan, chitosan alone and ulvan/chitosan polyelectrolyte membranes have been synthesised and characterised. The structure of the membranes was altered by the weight ratio of the polyion components. Fibrous and nanofibrous morphology was created, in accordance with a supramolecular self assembly. ATR-FTIR measurements suggested the presence of both polycationic chitosan and polyanionic ulvan in the polyelectrolyte membranes. The cytocompatibility of these new materials was examined by fluorescence microscopy. The results show that ulvan as well as ulvan/chitosan membranes promoted the attachment and proliferation of 7F2 osteoblasts and maintained the cell morphology and viability. Thus, ulvan and chitosan which possess unique properties might have high impact in biomedical applications as potential scaffold materials.

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1. Introduction

Ulvan is a complex anionic sulfated polysaccharide extracted from the cell-walls of green sea weeds (Ulvales, Chlorophyta). Sulfated, rhamnose, xylose, glucuronic and iduronic acids are the main constituents of ulvan. In general, the main disaccharide units that constitute the aldobiuronic acid blocks of ulvan, also reported as ulvanobiuronic acid 3-sulfate, are formed by a Type A_{3s} glucuronorhamnose and a Type B_{3s} iduronorhamnose, arranged in regular sequences within the heteropolymer chain (Lahaye & Robic, 2007) (Fig. 1). Ulvan has been reported as anticoagulant, antioxidant, antitumor and immune modulator. Furthermore it can lower the low-density lipoprotein cholesterol (LDL-cholesterol) thus reducing the atherogenic index (Lahaye & Robic, 2007; Morelli & Chiellini, 2010; Zhang et al., 2008).

Chitosan, a high molecular weight polysaccharide composed of β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units, is a deacetylated form of chitin. This natural cationic polymer, offers unique properties; it is biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional. Chitosan has been proven to accelerate wound-healing, stimulate the macrophage activity, and inhibit the growth of tumor cells and posses antimicrobial properties (Muzzarelli, 2009a, 2011).

Polyelectrolyte complexes involve anionic and cationic side chain reactions on their macromolecular backbone (Schwarz, Richau, & Paul, 1991). Therefore, ulvan and chitosan as oppositely charged macromolecules can potentially form a polyelectrolyte assembly. Chitosan complexes with the polyanionic sulfoethyl cellulose have been proposed as the most promising candidates for tailored wet wound dressings in chronic cases (Clasen, Wilhelms, & Kulicke, 2006). Chitosan/alginate films, cross-linked with genipin have been proven to be highly cell adhesive and can be suitable for use in tissue engineering and improved tissue–implant interfaces (Hillberg, Holmes, & Tabrizian, 2009; Muzzarelli, 2009b).

Besides, chitosan is known for its fiber and nanofiber ability (Agboh & Qin, 1996; Ohkawa, Cha, Kim, Nishida, & Yamamoto, 2004). Recently, the nanofiber ability of ulvan has also been reported (Toskas, Hund, et al., 2011). Parts of the novel combined ulvan/chitosan fibrous membrane alteration techniques are now covered by a patent (Toskas, Roussis, & Smyrniotopoulos, 2011). The present study has focused in the formation and evaluation of single ulvan, chitosan and ulvan/chitosan complex membranes by solvent casting. The morphology of the membranes

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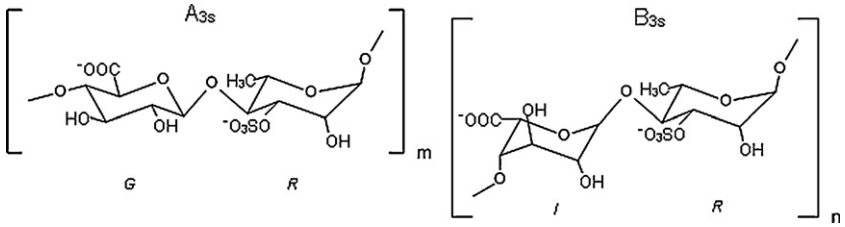

Fig. 1. The structure of the main disaccharide units in *Ulva* ulvan: A_{3s} consisting of [β-DGlcp A-(1,4)-α-L-Rhap 3s] and B_{3s} consisting of [α-L-Idop A-(1,4)-α-L-Rhap 3 s]; *G*: (1,4)-linked β-D-glucuronic acid; *R*: (1,4)-linked α-L-rhamnose-3-sulfate; *I*: (1,4)-linked α-L-iduronic acid.

was examined and followed by cell attachment experiments. The cytocompatibility of the natural anionic polysaccharide ulvan as well as that of the ulvan/chitosan polyelectrolyte complex are explored for the first time.

2. Materials and methods

2.1. Materials

2.1.1. Ulvan

The plant material of *Ulva rigida* was collected in Greece. The extraction and chemical analysis of ulvan was carried out at the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens (ATPH/MO/205), as previously described (Toskas, Hund, et al., 2011).

2.1.2. Ulvan characterisation

High performance size exclusion chromatography (HPSEC) revealed that the acidic polysaccharides had a molecular weight distribution from 30,580 to 59,950 Da.

NMR spectra were recorded using a Bruker DRX 400 spectrometer. Typical ulvan proton chemical shifts were observed (Electronic Supplementary Information (ESI), SF. 1) (Lahaye, Inizan, & Vigouroux, 1998). The infrared absorption spectrum of ulvan extract from *U. rigida* presented all characteristic absorbances typical for ulvan polysaccharides (Electronic Supplementary Information (ESI), SF. 2) (Toskas, Hund, et al., 2011).

2.1.3. Chitosan

Chitosan from crab shells with >75% of deacetylation (M_W = 200 kDa) was obtained from Sigma, Germany and used without further purification.

2.2. Membrane fabrication

2.2.1. Ulvan membrane

For the ulvan membranes preparation, optimised solutions were adjusted at 2.34 wt% of ulvan in a medium containing 15 mM H_3BO_3 and 7 mM $CaCl_2$ in a ratio of 60/40 (v/v) in deionized water. The solutions were stirred on a magnetic stir plate for at least 6 h until the formation of a uniform gel with pH 6.9. The initial dynamic viscosity of the 2.34% ulvan solution was measured 78,000 mPa s. Thin films (~35 μm) were prepared by evaporative casting of solutions by applying to a flat glass surface (Petri plate) and dried at controlled ambient conditions, of 20 °C temperature, 55% relative humidity for 48 h.

2.2.2. Chitosan membrane

Chitosan solutions were obtained with 2.0–3.0 wt% chitosan solubilised for 2 h in 15 mM H_3BO_3 and subsequent addition of up to 20 wt% total acetic acid concentration. The initial dynamic viscosity of these solutions ranged from 1000 to 3000 mPa s. The pH of the chitosan solutions was between 3.0 and 4.5.

2.2.3. Ulvan/chitosan membranes

The ulvan/chitosan polyelectrolyte membranes were prepared from the above described ulvan and chitosan solutions under vigorous stirring (Vortex) at various ratios for 10–30 min. For the morphology examinations, thin films (4–40 μm) were casted on glass Petri plates at controlled ambient conditions, as previously. The resulted films were washed thrice with Milli-Q water before drying once more. The pH of the so casted films was found near neutral, 6.7–6.9.

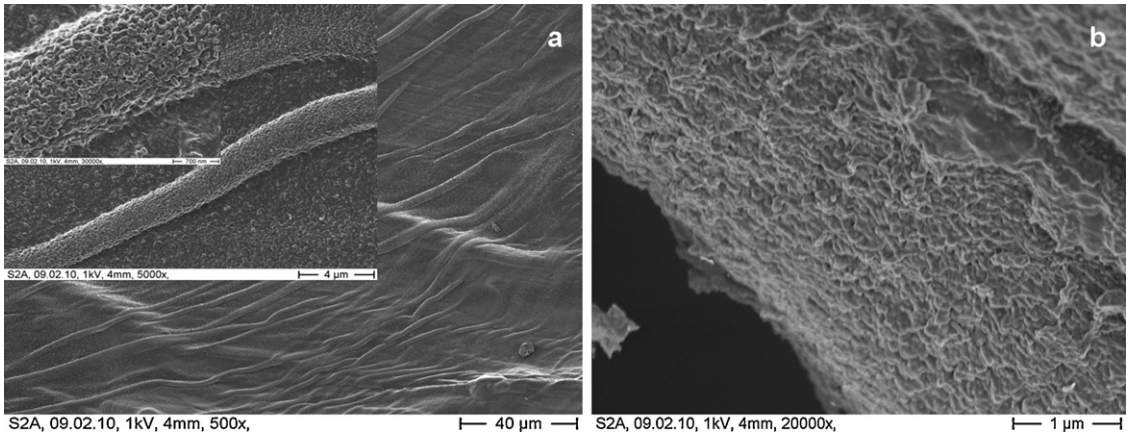

Fig. 2. (a) Rough surface with open pores on fibrous structured membranes from a ratio of ulvan/chitosan = 1:2 (magnification 500×; inserts: magnification 5000× and 30,000×). (b) Porous cross-section (magnification 20,000×).

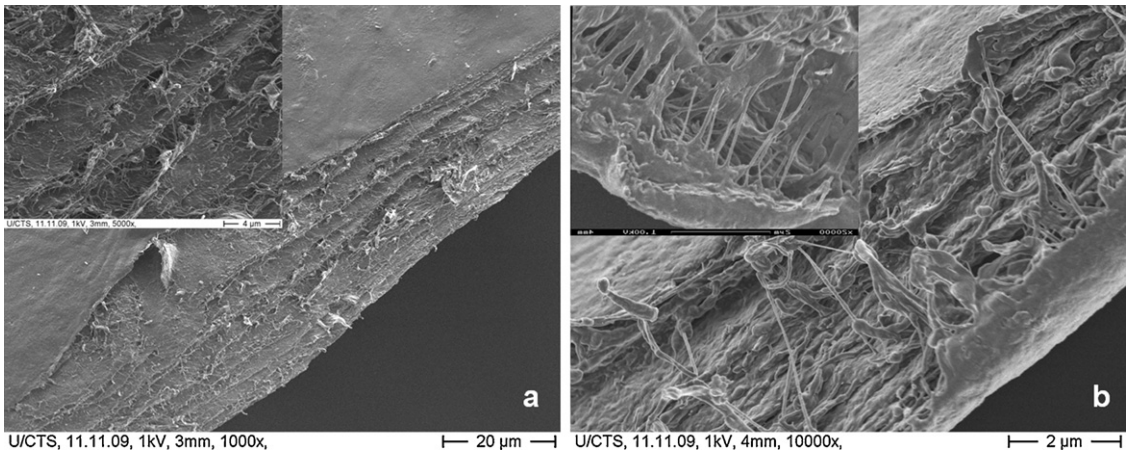

Fig. 3. (a) Nanofibrous self-assembled layer-by-layer structured membranes with flat surface and closed pores from a ratio of ulvan/chitosan = 5:8 (magnification 1000×; insert: magnification 5000×). (b) Cross-section showing nanofibers with diameters of 34–70 nm (magnification 10,000×; insert: magnification 50,000×).

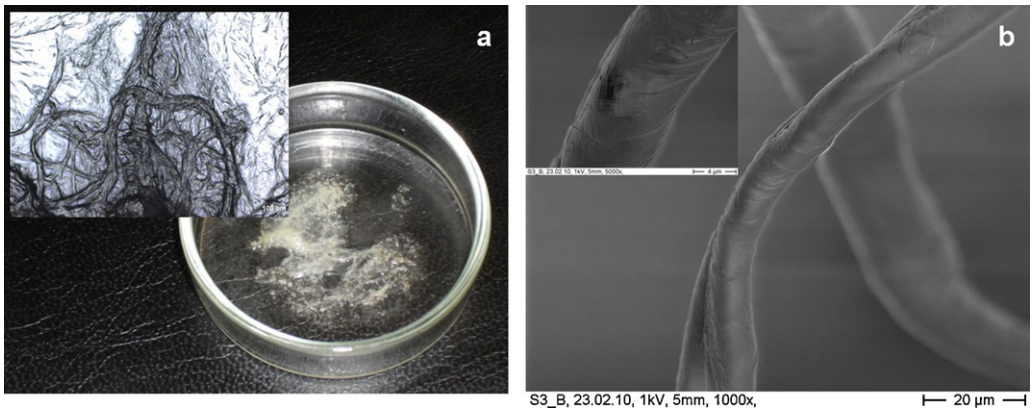

Fig. 4. (a) Photo of ulvan/chitosan microfibers formed *in situ* (insert: optical microscopy, scale 100 µm). (b) SEM image of microfibers having diameters of 7–8 µm (magnification 1000×; insert: magnification 5000×).

2.3. Characterization

A DSM 982 Gemini (Zeiss, Germany) Scanning Electron Microscope served for the examination of the morphology of the membranes. The as-casted membranes were dried and sputter-coated with silver/graphite. The samples were examined at an accelerating voltage of 1.0 kV and magnifications from 500 to 50,000.

Fluorescence microscopic images have been taken with a Zeiss Axioskop 2 FS mot equipped with both a Zeiss Plan Neofluar® 10×/0.3 and a Zeiss Plan-Apochromat® 20×/0.75. The excitation was performed by a 50 W-mercury lamp. Filter sets Zeiss Nr 1 (DAPI) as well as Zeiss Nr. 9 (Alexa Fluor 488®) have been used.

IR spectra were obtained by using the attenuated total reflection (ATR) method on a FTIR Bruker Tensor 27 spectrophotometer.

2.4. Cell culture and staining

Nanofibrous membranes for cell culture were prepared on titanium plates (10 mm diameter) by dip coating. The particular coating solutions consisted of the above described ulvan and chitosan solutions at various ratios. After coating, drying at 37 °C and 20% relative humidity was performed over 24 h in a climate chamber. The resulting membranes on titanium plates were sterilized by gamma-irradiation (25 kGy) before starting the cell culture experiments. Murine osteoblast-like cell line 7F2 was obtained from the American type Culture Collection (ATCC). Cells were expanded in

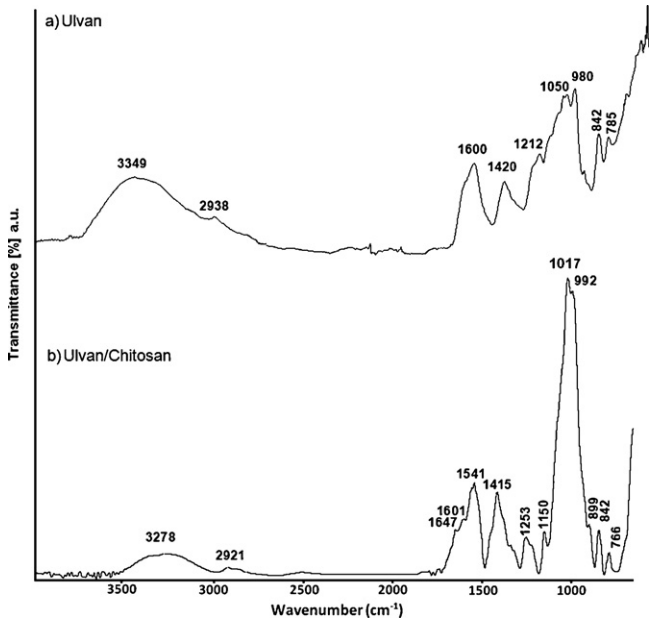

Fig. 5. ATR-FTIR spectra of (a) ulvan and (b) ulvan/chitosan film from a ratio of ulvan/chitosan = 5:8.

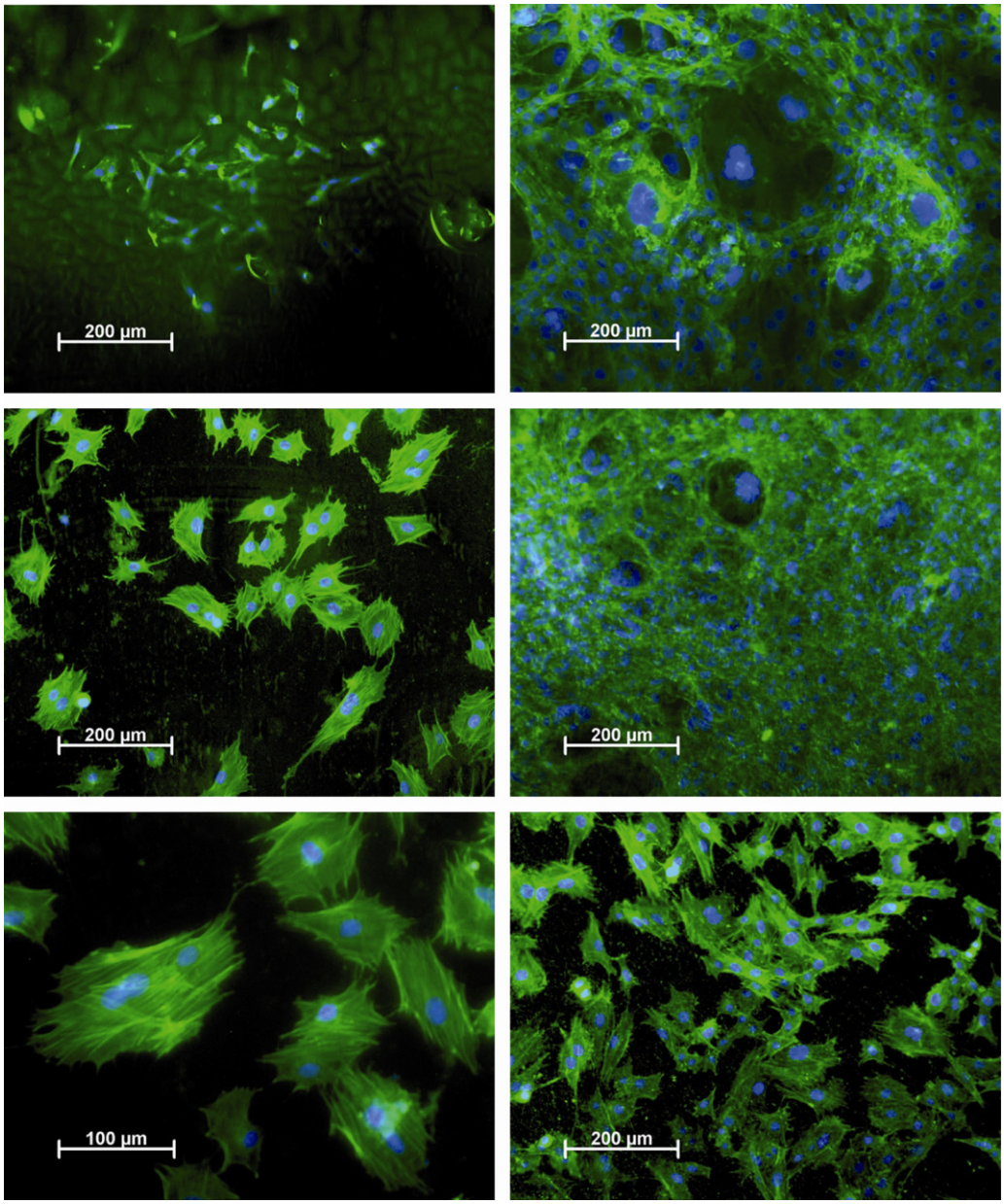

Fig. 6. Fluorescence microscopic images of 7F2-cells on (upper panel left) chitosan-film after 6 days culture time; (upper panel right) ulvan-film after 6 days; (middle panel left) ulvan/chitosan-film (4:5) after 1 day and (middle panel right) 6 days culture time; (lower panel left) ulvan/chitosan-film (4:5) after 6 days, more detailed image; (lower panel right) pure titanium after 1 day as a reference.

minimal essential medium (R-MEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and the antibiotics penicillin (100 U/mL) and streptomycin (100 U/mL) in a humidified atmosphere (37 °C, 7% CO_2). Medium and all supplements were obtained from Biochrom, Germany.

The membranes were placed in 48-well plates and soaked in cell culture medium for 24 h. After removing the medium, 40 μL of cell suspension (2500 cells per μL) was placed onto each sample. Cells were allowed to adhere for 30 min in the incubator before filling up the wells with additional medium. The medium was changed every second day.

In order to evaluate cell morphology, spreading, and growth using fluorescence microscopy actin cytoskeleton as well as the nuclei were stained after 1 day and 6 days. To that end, after washing and fixing, the cells were permeabilized with 0.2% Triton-X-100 in PBS and blocked with 1% bovine serum albumin (BSA, Sigma) for 30 min. Cytoskeletal actin was stained with AlexaFluor 488-Phalloidin (Invitrogen), and cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma).

3. Results and discussion

Ulvan is a lightly branched anionic polysaccharide containing two negatively charged groups, carboxylates and sulfate esters in the repeating disaccharides consisting of glucuronic or iduronic acid and rhamnose sulfate (Fig. 1). Glucuronic acid has a $pK_a = 3.28$, while chitosan which is soluble in water under acidic conditions has an intrinsic $pK_a = 6.3$. However, it has been found that the pH of the chitosan casted films is near neutral, regardless of the pH of solutions (Amin & Panhuis, 2011). Glucuronic and iduronic acids each contain one carboxylate ion, while the two rhamnoses each one sulfate ion. For the complete reaction of these four anionic groups, four protonated amine cations from the 75% deacetylated chitosan are needed. Taking in account the ulvan and chitosan molecular

weights of the monomers and the respective solution concentrations, we could calculate the optimum weight ratio, rating ulvan/chitosan = 4:5, which corresponds to the complete neutralization of the side chains forming the polyelectrolyte backbone. Nonetheless, in order to examine the respective structural effect on membranes, various ratios of the two polyelectrolytes were mixed. It was found that for a ratio of ulvan/chitosan = 1:2, the membrane surface presents a fibrous porous structure with pore diameters estimated by SEM to 23–58 nm (Fig. 2). When the ratio of ulvan/chitosan was approaching 2:3, the porosity is reduced but the nanofibrous structure is appearing (Fig. 3). Actually, one can observe at the membrane's cross-section, that the components have a self-assembled layer-by-layer construction (Fig. 3a), interconnected by nanofibers with diameters of 34–70 nm (Fig. 3b).

Concurrently to the membrane formation, some individual microfibers with diameters of 7–8 μm are formed *in situ*. They can be also seen by optical microscopy (Fig. 4a), and present by SEM the characteristic for the polysaccharides multilayer surface morphology (Fig. 4b).

The composition of the films was examined by ATR-FTIR spectroscopy. The infrared absorption spectrum of ulvan film is shown in Fig. 5a. All characteristic absorbances typical for ulvan polysaccharides were observed and are reported in Supplementary Information (Pengzhan et al., 2003; Ray & Lahaye, 1995; Robic, Bertrand, Sassi, Lerat, & Lahaye, 2009; Toskas, Hund, et al., 2011). The bands at wavelengths between 980 and 1050 cm^{-1} can be attributed to the B—OH and B—O groups of the boric acid (Chetri, Dass, & Sarma, 2007).

The FT-IR spectrum of an ulvan/chitosan membrane, composed with solutions of the two components at weight ratio of ulvan/chitosan = 5:8, is shown in Fig. 5b. The characteristic for the chitosan carbonyl, C=O—NHR absorption band was observed at 1648 cm^{-1}, assigned to the amide I vibration. Also, the ulvan characteristic sulfate esters band at 1253 cm^{-1} is observed together with the two bands at 845 and 785 cm^{-1}. Carboxylate groups show two bands: a broad asymmetrical stretching band at 1600–1650 cm^{-1} and a symmetric stretching band at 1415 cm^{-1}, resulting from both constituents' ulvan and chitosan.

Beside the bands mentioned above, there are three main features in the ulvan/chitosan spectrum (Fig. 5b): *A*. A new peak is appeared at 1540 cm^{-1} assigned to one of the $-NH_3^+$ vibrational modes, the other overlapping with the amide I band. *B*. The bands at 990–1017 cm^{-1} are accentuated, showing stronger skeletal vibrations of C—O stretching caused by both components. Yet, an enhanced borate involvement in an ionic assembly within the PEC formation incorporating hydroxyl groups, as it was previously postulated for the ulvan-poly (vinyl alcohol) (PVA) fibers (Toskas, Hund, et al., 2011), can be proposed. *C*. The shift of the weaker O—H stretching mode to lower wavelength, centered at 3278 cm^{-1} satisfies not only the absorbance of N—H stretch of chitosan, but also supports the before cited argument on the borate esters role. Thus, the ATR-FTIR spectrum of the ulvan/chitosan membrane not only exhibits the characteristic signals of both materials, but induces a strong polyelectrolyte matrix through ionic and borate-hydroxyl covalent bonds.

The complex nanofibrous membranes were subsequently examined in respect to their cellular compatibility and compared to the solely ulvan or chitosan membranes.

The fluorescence images (Fig. 6) show clearly the advantages of ulvan/chitosan mixed film over pure chitosan as substrate for 7F2 osteoblasts. Also in case of a pure ulvan film, the 7F2-layer after 6 days culture time shows a lower confluence than that on the mixed film. The mixed films show an excellent attachment and spreading of the cells even after 1 day culture time as one can see in the corresponding images in Fig. 6. It resembles the situation on pure titanium, a very suitable substrate for osteoblasts. After 6 days culture time on ulvan/chitosan-films a completely confluent layer can be observed, *i.e.* the 7F2-cells show an excellent proliferation on that substrate. From this point view, the ulvan/chitosan-films are suitable substrates for the cultivation of osteoblasts.

4. Conclusions

The combination of two polymers of oppositely charged backbone, such as the anionic ulvan and the cationic chitosan, leads through electrostatic interactions to the formation of supramolecular structures and stabilised membranes. Variation on porosity can be altered by changing the weight ratio of the two polysaccharides. The nanofibrous structure of these constructs mimicking the fibrous part of the extracellular matrix structure might be responsible for the excellent attachment of the 7F2 osteoblasts. The cytocompatibility of ulvan, alone or in polyelectrolyte complex with chitosan is for the first time reported. The relative collective properties of the ulvan/chitosan membranes provide a good basis for the development of scaffolds from these materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.04.045.

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