

# Cellulose-based materials as scaffolds for tissue engineering

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Received: 5 April 2013 / Accepted: 19 July 2013 / Published online: 28 July 2013  
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**Abstract** Two types of cellulose-based materials, 6-carboxycellulose with 2.1 or 6.6 wt% of –COOH groups, were prepared and tested for potential use in tissue engineering. The materials were functionalized with arginine, i.e. an amino acid with a basic side chain, or with chitosan, in order to balance the relatively acid character of oxidized cellulose molecules, and were seeded with vascular smooth muscle cells (VSMC). The cell adhesion and growth were then evaluated directly on the materials, and also on the underlying polystyrene culture dishes. Of these two types of studied materials, 6-carboxycellulose with 2.1 wt% of –COOH groups was more appropriate for cell colonization. The cells on this material achieved an elongated shape, while they were spherical in shape on the other materials. The number of cells and the concentration (per mg of protein) of contractile proteins alpha-actin and SM1 and SM2 myosins, i.e.

markers of the phenotypic maturation of VSMC, were also significantly higher on this material. Functionalization of the material with arginine and chitosan further improved the phenotypic maturation of VSMC. Chitosan also improved the adhesion and growth of these cells. In comparison with the control polystyrene dishes, the proliferation of cells on our cellulose-based materials was relatively low. This suggests that these materials can be used in applications where high proliferation activity of cells is not desirable, e.g. proliferation of VSMC on vascular prostheses. Alternatively, the cell proliferation might be enhanced by another more efficient modification, which would require further research.

**Keywords** Polysaccharide materials · Oxidized cellulose · Vascular smooth muscle cells · Cell differentiation · Contractile phenotype · Chitosan

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## Introduction

Cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand  $\beta(1 \rightarrow 4)$  linked D-glucose units. It was discovered and isolated from green plants by Payen (1838); for a review, see O'Sullivan (1997). It is the most abundant biopolymer on Earth, synthesized by grasses, woody plants, many forms of algae, fungi and some species of bacteria, namely *Acetobacter xylinum*. Bacterial cellulose is identical to plant cellulose in chemical structure, but it can be produced without contaminant molecules, such as lignin and hemicelluloses, and does not require intensive purification processes. In addition, it is remarkable for its mechanical strength and biocompatibility, so it has often been applied in tissue engineering (Petersen and Gatenholm 2011). For example, tubes 4 mm in diameter made of bacterial cellulose showed compliance similar to that of human saphenous vein and higher than expanded polytetrafluorethylene (ePTFE) and polyethylene terephthalate (PET), i.e. synthetic polymers currently used for fabricating vascular replacements. At the same time, they supported the development of a layer of vascular smooth muscle cells. These tubes were therefore considered to be suitable for engineering small-caliber vascular grafts (Zahedmanesh et al. 2011).

However, plant cellulose also holds promise for tissue engineering and for other biomedical applications (Kolarova et al. 2013). Various types and modifications of this cellulose are under extensive scientific research, and their biocompatibility and bioactivity have been tested with various cell types (Mikulikova et al. 2008). Regenerated cellulose (i.e., purified cellulose in which short fibers coming from trees are chemically converted into the long fine fibers used in textiles and non-wovens) has been used for constructing three-dimensional tissue with capillaries (Ko and Iwata 2001) and, together with cellulose acetate, also for cardiac tissue engineering (Entcheva et al. 2004). Non-woven cellulose fabrics (Müller et al. 2006) and injectable cellulose-based hydrogels (Vinatier et al. 2009) have been successfully tested as carriers for chondrocytes for cartilage regeneration in vitro and in vivo. Viscose cellulose sponges have been investigated for their biocompatibility with the bone tissue (Mårtson et al. 1998), and also as scaffolds for cartilage tissue engineering (Pulkkinen et al. 2006).

Ideal scaffolds used in advanced tissue engineering should be constructed from resorbable materials that

degrade in the organism and are gradually replaced by natural extracellular matrix (Collins and Birkinshaw 2013). However, cellulose in the human organism behaves as a non-degradable or very slowly degradable material. For example, the degradation time of viscose cellulose sponges implanted subcutaneously into rats was longer than 60 weeks (Mårtson et al. 1999). This very slow degradability of cellulose is due to the absence of enzymes that attack the  $\beta(1 \rightarrow 4)$  linkage (these enzymes are present in microbial and fungal cells; for a review, see Mårtson et al. 1999). An efficient method for inducing degradability of cellulose is its oxidation. Oxidized cellulose can be generated by various techniques and by various oxidizing agents, such as  $\text{NaClO}_2$ ,  $\text{CCl}_4$ , nitrogen oxides or free nitroxyl radicals (TEMPO oxidation; Kumar and Yang 2002; Zimnitsky et al. 2004; Saito et al. 2007). Oxidized cellulose is degradable by hydrolysis, by mediated hydrolytic enzymes present in the serum supplement of cell culture media in vitro, and in vivo, in macrophages (Dimitrijevič et al. 1990a, b).

Cellulose oxidation induces conversion of the glucose residues to glucuronic acid residues containing  $-\text{COOH}$  groups. The concentration of these groups modulates not only the degradation time of cellulose, but also its pH, its swelling in a water environment, mechanical stability, drug loading efficiency and other behavior of the material (Zhu et al. 2001). In addition, the  $-\text{COOH}$  groups, which are polar and negatively charged, can be used for functionalizing the oxidized cellulose with various biomolecules (Zimnitsky et al. 2005, 2006).

Oxidized cellulose has been widely used for many years as a wound healing material with excellent properties, such as high absorbability, antibacterial and antiviral properties, and non-toxic and anti-adhesive effects (Mueller et al. 2000; Jeschke et al. 2005; Bassetto et al. 2008; Vytrasova et al. 2008). Due to its ability to initiate or accelerate blood coagulation at the site where it is applied, oxidized cellulose has been used as a hemostatic material (Masova et al. 2003; Schonauer et al. 2004). Oxidized cellulose is also promising as a carrier for controlled drug delivery (Zhu et al. 2001). Oxidized dialdehyde cellulose has been applied as a biodegradable scaffold for tissue engineering (Roychowdhury and Kumar 2006), e.g. of vocal fold lamina propria (Roychowdhury et al. 2009).

In this study, we investigate the adhesion, growth and phenotypic maturation of vascular smooth muscle cells (VSMC) on two types of cellulose materials for

potential use in soft tissue engineering: oxidized 6-carboxycellulose with 2.1 wt% of –COOH groups (2.1) and oxidized 6-carboxycellulose with 6.6 wt% of –COOH groups (6.6). In order to compensate the relatively acid character of oxidized cellulose molecules (Nagamatsu et al. 1997), the materials were functionalized with arginine, i.e. an amino acid with a basic side chain (Harms et al. 2011), or with chitosan, a linear cationic polysaccharide which has been shown to improve the adhesion and growth of several types of cells, e.g. vascular endothelial cells (Rubenstein et al. 2010), skin keratinocytes (Kingkaew et al. 2010) and 3T3 fibroblasts (Weltrowski et al. 2012) on cellulose-based scaffolds.

## Experimental

### Oxidation of cellulose scaffolds

Pure multifilament viscose silk yarn was used for knitting the starting cellulose tissue. Special attention was paid to avoiding use of any lubricants during knitting in order to protect the cellulose tissue material against hydrophobic substances that would be difficult to remove. The tissues were developed on demand for purposes of this study. Cellulose tissues were oxidized in an organic solvent. Perfluorosol PFS-1 was used as the organic solvent, and nitrogen tetroxide was used as the oxidizing agent. The mixture of a solution of nitrogen tetroxide (10 vol%) in PFS-1 (90 vol%) was prepared. To obtain a desirable degree of oxidation (1–22 %), the process was performed at room temperature for designated times. After the oxidation, the washing process in demineralized water was performed, as follows: in demineralized water, in a water solution of isopropyl alcohol in a concentration of 50 wt%, and in 100 % isopropyl alcohol. Drying was carried out in the open air, at room temperature. Biofunctionalized cellulose scaffolds were prepared from oxidized cellulose containing 2.1 or 6.6 wt% of –COOH groups. The content of –COOH groups was determined by the standard acidobasic titrimetric method for oxidized cellulose according to the United States Pharmacopoeia.

### Biofunctionalization of cellulose scaffolds

Cellulose-based materials (listed above) in the form of woven fibrous scaffolds were exposed to solutions of

arginine or chitosan (USA, Sigma-Aldrich) for 2 h at room temperature (20 °C). The quantities of particular substances used for preparing the solutions are summarized in Table 1. The samples were then washed 2 times for 2 h in isopropyl alcohol and air-dried.

### Characterization of the materials

#### Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) with the VEGA 3 LMU, TESCAN (CZ) device with voltage 20 eV was used for studying the surface morphology. The samples were covered with gold (the thickness of a layer was ca 15 nm) in a sputter coater with glow discharge.

#### X-ray photoelectron spectroscopy (XPS)

XPS spectra were obtained on the Omicron Nanotechnology ESCAProbeP spectrometer. The dimensions of the exposed and analyzed area were  $2 \times 3 \text{ mm}^2$ . The measuring conditions were as follows, monochromated X-ray source at 1486.7 eV with measuring step 0.05 eV and take-off angle 0 with respect to the surface normal, penetration depth 1 nm. Measurements were made in an ultralight vacuum. The evaluation of the acquired spectra was carried out by CasaXPS code (Svorcik et al. 2009).

#### Fourier transform infrared spectroscopy (FTIR)

The changes in chemical structure were examined by FTIR on a Bruker NICOLET 6700 ISF 66/V spectrometer (Thermo, Nicolet, USA) with a GladiATR

**Table 1** Quantity of ingredients of the arginine (arg) or chitosan (chit) solution that was used for functionalization of primal materials (2.1, 6.6: 6-carboxycellulose with 2.1 and 6.6 wt% of –COOH groups, respectively)

Prepared biofunctionalized material	Primal textile material (g)	Arginine or chitosan (g)	Acetic acid (ml)	Distilled H <sub>2</sub> O (ml)
2.1+arg	6	1.48 (arg)	0	150
2.1+chit	6	6 (chit)	2.5	500
6.6+arg	6	4.76 (arg)	0	150
6.6+chit	6	6 (chit)	2.5	500

diamond ATR extension. The differential FTIR spectra presented in this study were determined by subtracting the FTIR spectra of non-functionalized samples from those of functionalized samples.

#### *pH measurements*

The samples were cut out on circles 1 cm in diameter. The samples were immersed into distilled water at laboratory temperature, volume 50 ml. The pH values were written down every 30 s for a period of 40 min. The solutions were remeasured after 48 h. The pH was examined using an Orion Star A211 pH meter.

#### Cells and culture conditions

##### *Evaluation of cell adhesion and growth*

Samples of cellulose fabrics 2 × 2 cm in size were sterilized by UV light for 1 h on each side. Sterilization by UV light appeared to be the most appropriate approach for this kind of material, as sterilization in ethanol or in an autoclave in our preliminary experiments caused morphological, chemical and mechanical instability of the samples. After sterilization, the samples were inserted into 12-well culture plates (well diameter 2.2 cm; TPP, Switzerland). To prevent the samples floating in the culture medium, they were fixed to the well bottoms with plastic rings, and then seeded with vascular smooth muscle cells, derived from rat aorta by an explantation method (Bacakova et al. 1997). The cells (passage 4) were seeded at a initial density of 60,000 cells/well (i.e., about 16,000 cells/cm<sup>2</sup>). The cells were cultured in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. No. D5648) supplemented with 10 % fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia) at 37 °C in a humidified atmosphere containing 5 % of CO<sub>2</sub> in the air. Materials with 6.6 wt% of –COOH groups, especially those non-modified with arginine and chitosan, caused an excessive decrease in the pH of the culture medium, indicated by the phenol red pH indicator contained in the medium, which changed the colour of the medium from pink to yellow as early as a few hours after seeding. The medium was therefore replaced by a fresh medium, and after it had been replaced the same effect did not recur.

On the 2nd, 4th and 7th day after seeding, the cells were rinsed with phosphate-buffered saline (PBS; Sigma–Aldrich), detached from the materials by a trypsin–EDTA solution (Sigma, USA, Cat. No. T4174) and counted using a Bürker haemocytometer. Two independent samples were used for each time interval and experimental group.

##### *Evaluation of the cell viability and morphology*

The cells on the materials and also on the underlying polystyrene dishes were stained with the viability/cytotoxicity (LIVE/DEAD) kit for mammalian cells (Invitrogen, Molecular Probes, USA) on days 2, 4 and 7 according to the manufacturer's protocol. After being rinsed with PBS, the cells were incubated (10 min, 20 °C) in a mixture of calcein AM, which detects the esterase activity in living cells and produces a green fluorescence, and ethidium homodimer-1, which labels the dead cells by penetrating into them through their damaged membrane and generating a red fluorescence. Microphotographs of the cells were taken using a conventional fluorescence microscope (Olympus IX 50, Japan) complemented by a digital camera (DP 70, Japan).

##### *Immunofluorescence staining of alpha-actin and SM1 and SM2 myosins*

The cellulose-based scaffolds were cut into square pieces (1 × 1 cm) and sterilized by UV light. All materials were washed in PBS and in the culture medium (mentioned above) in order to increase the pH of the oxidized samples (particularly materials with 6.6 wt% of –COOH groups), and were fixed to the well bottoms with plastic rings. Twenty-four-well culture plates (well diameter 1.5 cm; TPP, Switzerland) were used for cell cultivation, and vascular smooth muscle cells (passage 8) were seeded at a density of 30,000 cells/well (i.e., about 17,000 cells/cm<sup>2</sup>). The cells were cultured in DMEM, as mentioned above (1 ml/well; Sigma, USA, Cat. No. D5648) supplemented with 10 % FBS (Sebak GmbH, Aidenbach, Germany) and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia) at 37 °C in a humidified atmosphere containing 5 % of CO<sub>2</sub> in the air.

The contractile proteins alpha-actin and SM1 and SM2 myosins, important markers of the phenotypic maturation of VSMC towards the contractile phenotype, were visualized by immunofluorescence. On the

7th day after seeding, the cells were fixed with 70 % methanol (−20 °C, 10–20 min). In order to block non-specific binding sites and to permeabilize the cells, the samples were pretreated with 1 % bovine serum albumin and 0.1 % Triton X-100 (Sigma) in PBS for 20 min at room temperature. Then the cells were incubated with the following primary antibodies: monoclonal anti-alpha smooth muscle actin (clone 1A4, Cat. No. A2547, Sigma) and monoclonal anti-myosin (clone hSM-V, Cat. No. M7786, Sigma), which were diluted in PBS to achieve concentrations of 1:200 (anti-alpha actin) and 1:100 (anti-myosin). The cells were kept at 4 °C in the antibody solutions overnight. After the cells had been rinsed with PBS, the secondary antibody, i.e. F(ab')<sub>2</sub> fragment of goat anti-mouse IgG (H + L) conjugated with Alexa Fluor<sup>®</sup> 488 and diluted in PBS to a concentration of 1:400, was added to both samples for 1 h (20 °C). The cells were then evaluated using a fluorescence microscope (Olympus IX 71, Japan) equipped with a digital camera (DP 71, Japan).

#### *Enzyme-linked immunosorbent assay (ELISA) of alpha-actin and SM1 and SM2 myosins*

The materials were inserted into 6-well culture plates (TPP, Switzerland, diameter 3.4 cm) and seeded with VSMC at an initial density of 200,000 cells/well (about 22,000 cells/cm<sup>2</sup>). On day 7 after seeding, the cells were detached from the materials and also from the underlying polystyrene dishes, using the trypsin–EDTA solution mentioned above, and were counted in a Bürker haemocytometer.

The cells were resuspended in distilled and deionized water and homogenized by ultrasonication (UP100H Ultrasonic Processor, Schoeller) for 20 s. The total content of protein was measured using the modified Lowry method (Lowry et al. 1951). Aliquots of the cell homogenates were diluted to the same concentrations (50 µg of total protein/ml). The aliquots were further used in 3 different concentrations (1, 5, 10 µg/ml), and were adsorbed on 96-well microtiter plates (50 µl of water with protein/well, Maxisorp, NUNC) overnight at 4 °C. After being washed twice with PBS, the non-specific binding sites were blocked by 0.02 % gelatine in PBS (100 µl/well, 1 h). Then the following steps were applied: (1) 1 % Tween<sup>®</sup> 20 (Sigma) for 20 min (100 µl/well), (2) washing in PBS, (3) washing twice in PBS with 0.1 %

Triton X-100, (4) washing in PBS. After that, the primary antibodies (the same as used for immunofluorescence staining), i.e. monoclonal anti-alpha actin and monoclonal anti-myosin (both diluted in PBS to concentration 1:200) were applied for 1 h (50 µl/well). Subsequent washing in PBS with 0.05 % Tween (3 times) was followed by applying the secondary antibody goat anti-mouse IgG conjugated with peroxidase (Cat. No. A3682, Sigma) for 45 min. After washing in PBS with 0.05 % Tween (3 times) again, a chromogen was applied (100 µl/well), and the plates were left for 10–30 min in the dark. The chromogen was prepared by mixing the following chemicals: (1) 10 ml of 0.1 M Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, (2) 5 mg of orthophenylenediamine dihydrochloride, and (3) 17 µl of 30 % H<sub>2</sub>O<sub>2</sub>. To stop the chromogen-protein reaction, 2 M H<sub>2</sub>SO<sub>4</sub> was used (50 µl/well). The absorbances were measured using a Versa Max ELISA Reader at 490 nm wavelength, and were expressed as a percentage of the control value obtained from cells growing on PS dishes with no cellulose material.

#### Statistical analysis

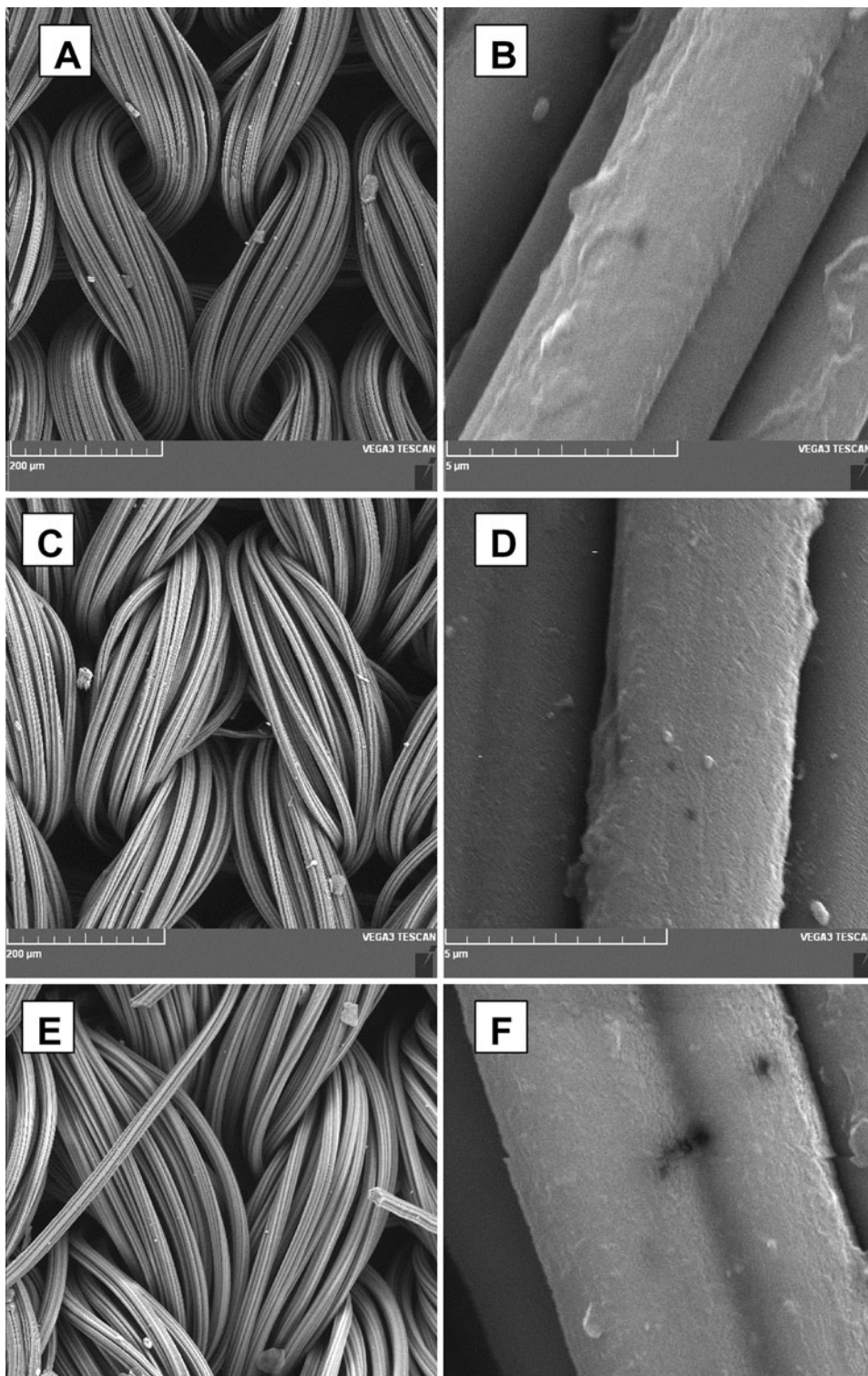
The quantitative data was presented as mean ± S.E.M. (Standard Error of Mean) from 36 measurements. Multiple comparison procedures were performed by the One Way Analysis of Variance (ANOVA), Student–Newman–Keuls method, using SigmaStat software (Jandel Corp. USA). *P* values equal to or <0.05 were considered significant.

## Results and discussion

### Surface morphology

SEM images of the cellulose-based samples are shown in Fig. 1. The fibers of 6-carboxycellulose are relatively smooth, knitted into a thick fabric with a typical multi-lobe cross-section due to the production process. A single fiber has an average width of 20 µm. Figure 1b, d and f show detailed images of single lobes. The average width of one lobe does not exceed 5 µm. There are no significant differences among the samples of 6-carboxycellulose with 2.1 or 6.6 wt% of –COOH groups. The images of the functionalized samples look similar to the previous images.



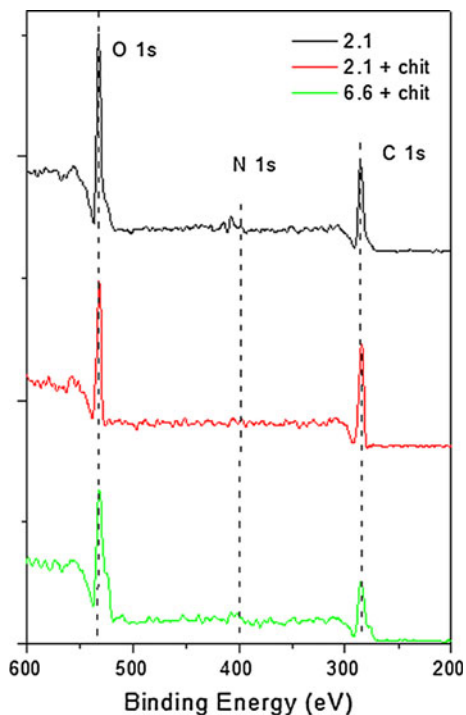


**Fig. 1** SEM images of non-oxidized 6-carboxycellulose (a, b), 6-carboxycellulose with 2.1 wt% (c, d) and 6.6 wt% (e, f) of -COOH groups. Bar = 200 μm (a, c, e) and 5 μm (b, d, f)

## Chemical analysis

Figure 2 shows the XPS spectra of chitosan-functionalized samples of 6-carboxycellulose with 2.1 or 6.6 wt% of  $-\text{COOH}$  groups. The nitrogen content in the case of chitosan corresponds to the line at 398.5 eV. This line can be seen clearly in both spectra. The nitrogen content was detected in the amount of 5.1 at% for 2.1 6-carboxycellulose functionalized with chitosan and 5.6 at% for 6.6 6-carboxycellulose functionalized with chitosan.

Both arginine- and chitosan-functionalized samples were further analyzed by FTIR spectroscopy. Fig. 3 shows that both samples contain peaks typical for arginine (2 912, 2 843 v valence vibrations of CH in  $-\text{CH}_2$  symmetric and asymmetric, 1 594 vibration of amidic group  $-\text{CO}-\text{NH}-$  and 1 400  $\text{cm}^{-1}$   $-\Delta-\text{CH}_2$  deformation vibration for samples functionalized by arginine) (Petrosyan and Sukiasyan 2008) and for chitosan (2 912, 2 843 v valence vibrations of CH in  $-\text{CH}_2$  symmetric and asymmetric, 1 598 vibration of amidic group  $-\text{CO}-\text{NH}-$ , 1 539 product of oxidation, 1 061—glycosidic bond and 987  $\text{cm}^{-1}$  hydroxyl)

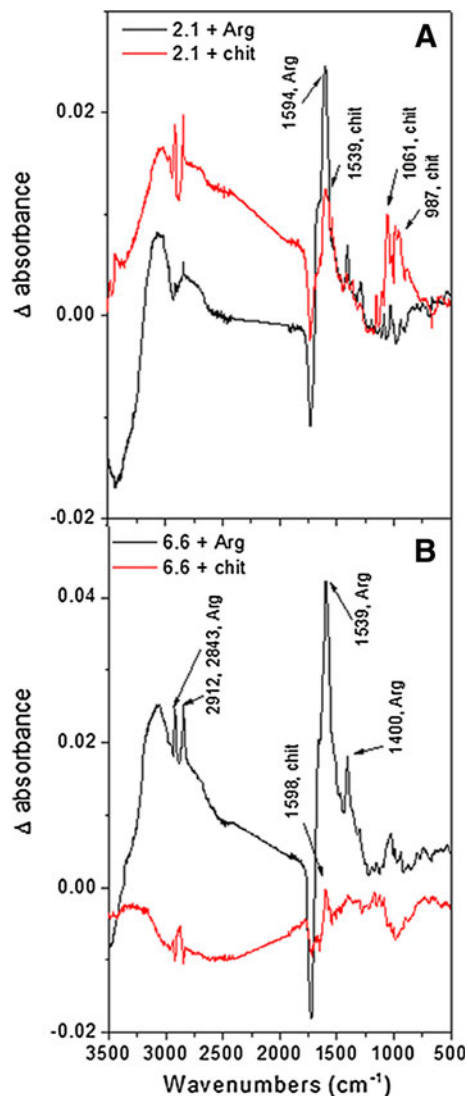


**Fig. 2** XPS spectra of 6-carboxycellulose with 2.1 wt% of  $-\text{COOH}$  groups (non-functionalized and functionalized with chitosan) and 6.6 wt% of  $-\text{COOH}$  groups (functionalized with chitosan)

group for samples functionalized by chitosan) (Lawrie et al. 2007).

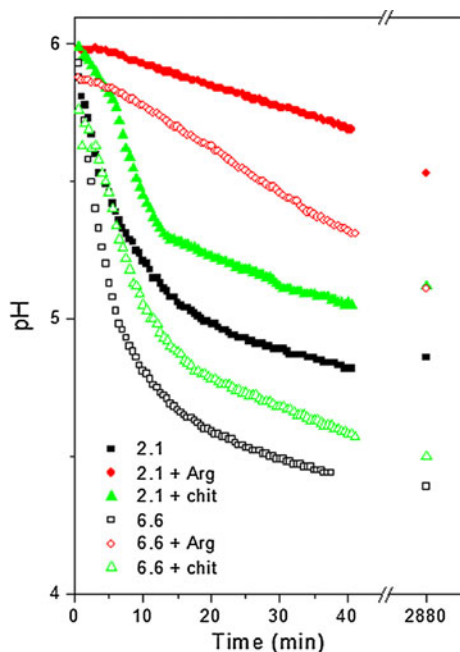
## pH Measurements

For the purposes of biological experiments, it is useful to know the behavior of the samples in aqueous solutions. When carboxycellulose is suspended in water, no appreciable solvation takes place, even after several weeks at 37 °C, but a fall in pH is observed. In



**Fig. 3** FTIR spectra of 6-carboxycellulose with 2.1 (a) and 6.6 wt% of  $-\text{COOH}$  groups (b) functionalized with chitosan or arginine. The resultant spectra were obtained by subtracting a control scan (i.e., without arginine or chitosan) and a scan of the material modified with arginine or chitosan

Fig. 4, the dependence of the pH values on the immersion time in distilled water can be observed. Samples of 6-carboxycellulose with 6.6 wt% of  $-COOH$  groups show a more significant decrease in the pH values than 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups, owing to the higher amount of carboxyl groups. The pH values of solutions with oxidized 6-carboxycellulose show a rapid decrease in the pH values during the first 15 min. After 45 min, the pH values are stable and the slightly acid environment (pH value under 5) prevents both materials dissolving. This is in accordance with the following statement that oxidized 6-carboxycellulose shows a polyanionic character immediately after coming in touch with a weak base. Continual titration is therefore needed to retain a stable pH value. A similar phenomenon can be observed in the biological system (in vivo) because of the natural buffering ability of the organism. Oxidized cellulose implanted in the body of a laboratory animal undergoes immediate degradation up to oligomers, which are then hydrolyzed to smaller fragments and finally to D-glucuronic acid, D-glucose (Dimitrijevič et al.



**Fig. 4** Dependence of the pH values on time of immersion in distilled water of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups (non-functionalized and functionalized with arginine and chitosan) and 6.6 wt% of  $-COOH$  groups (non-functionalized and functionalized with arginine or chitosan)

1990a, b). Higher pH values in arginine and chitosan-functionalized samples can be caused by the presence of amino groups in the samples. Arginine (Arg) is an ionizable amino acid. It is generally known that the antibacterial activity of chitosan is enhanced by lowering the pH values (Tsai and Su 1999). Accordingly, it can be presumed that the naturally acidic character of oxidized 6-carboxycellulose will support the antibacterial effectiveness of chitosan bound on the surface of the oxidized 6-carboxycellulose.

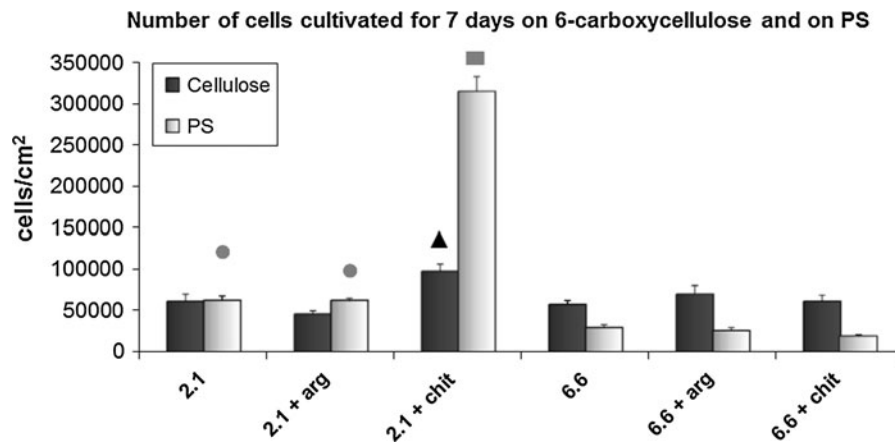
#### Adhesion and growth of VSMC on cellulose-based materials

In this study, we investigated the biocompatibility of 6-carboxycellulose with 2.1 or 6 wt% of  $-COOH$  groups for its potential use as a scaffold in tissue engineering. The materials were tested as non-modified and as modified with arginine and chitosan. Positively charged amine groups in these biomolecules were expected to support the adsorption of cell adhesion-mediating molecules and cell adhesion. At the same time, amino acids with the basic side chain (such as lysin, arginine), as well as chitosan, were assumed to balance the relatively acid character of oxidized cellulose molecules (Nagamatsu et al. 1997). Although this balancing effect was more apparent in samples functionalized with arginine than in samples functionalized with chitosan, the adhesion and subsequent growth were improved more apparently on the materials functionalized with chitosan.

On day 2 after seeding, the number of VSMC adhered to 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups and 6.6 wt% of  $-COOH$  groups was relatively low ( $2\,850 \pm 390$  cells/cm<sup>2</sup> and  $2\,920 \pm 400$  cells/cm<sup>2</sup>, respectively). For comparison, the initial seeding density was 16 000 cells/cm<sup>2</sup>, and on the bottoms of the control polystyrene wells without materials, the cell population density was  $8\,820 \pm 760$  cells/cm<sup>2</sup>. After functionalization with arginine, the cell population density on both types of 6-carboxycellulose remained similar to the population density on the non-modified materials. However, after functionalization with chitosan, the cell population density increased to  $4\,380 \pm 460$  and  $4\,380 \pm 520$  cells/cm<sup>2</sup> on 6-carboxycellulose with 2.1 and 6.6 wt% of  $-COOH$  groups, respectively.

On day 4 after seeding, the cell numbers on all cellulose-based samples became slightly higher,





**Fig. 5** Number of VSMC on day 7 after seeding on 6-carboxycellulose with 2.1 or 6.6 wt% of  $-COOH$  groups (2.1 and 6.6, respectively), and on the bottoms of polystyrene culture dishes (PS) in the presence of these materials. The materials were functionalized with chitosan (*chit*) or arginine (*arg*). Mean  $\pm$  S.E.M. from 18 measurements from 6 independent samples for each experimental group. ANOVA, Student–

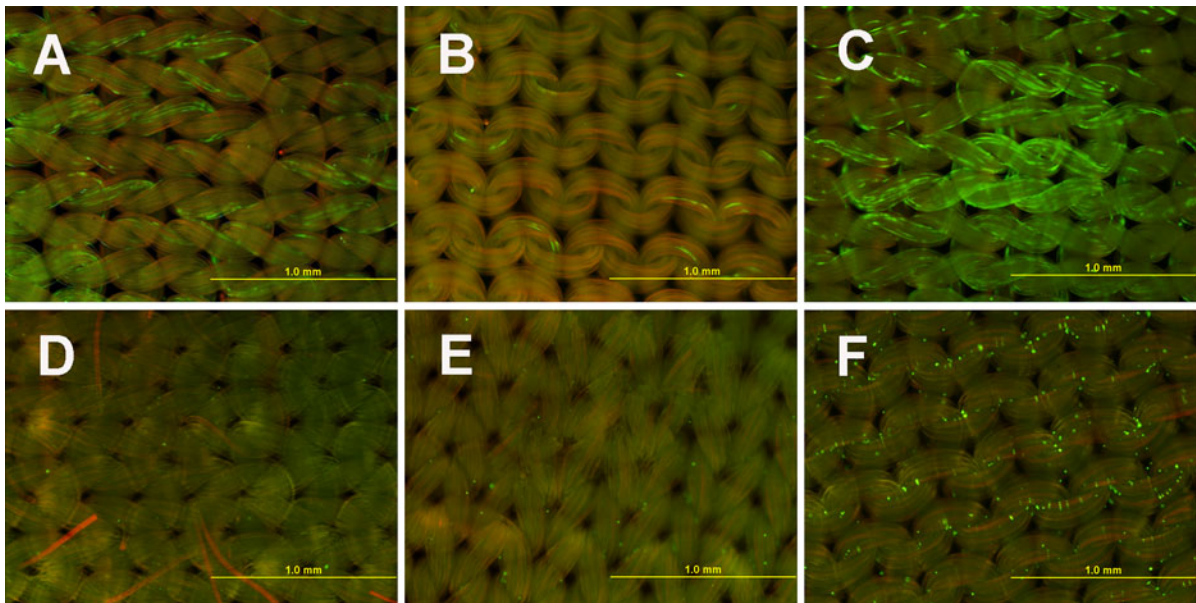
Newman–Keuls method applied for multiple comparisons. Statistical significance ( $p \leq 0.05$ ): *filled square* value in 2.1+chit (PS) is significantly higher than in all other samples (PS), *filled circle* values in 2.1 and 2.1+arg (PS) are significantly higher than in all other samples (PS), except 2.1+chit (PS), *filled triangle* value in 2.1+chit (Cellulose) is significantly higher than in all other samples

ranging from  $4\,510 \pm 530$  to  $7\,010 \pm 750$  cells/cm<sup>2</sup>, while on the control polystyrene wells, the cell population density reached  $61\,390 \pm 3\,060$  cells/cm<sup>2</sup>. The influence of functionalization with arginine and chitosan on the cell number was similar as on day 1.

On day 7, the increase in cell numbers on the cellulose-based materials was more apparent. The cell population densities on these materials ranged from  $46\,200 \pm 4\,590$  to  $97\,830 \pm 8\,840$  cells/cm<sup>2</sup>. The highest numbers were achieved on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups modified with chitosan. A relatively low cell number was found on 6-carboxycellulose with 6.6 wt% of  $-COOH$  groups, and functionalization with Arg and chitosan did not improve the cell growth on these materials (Fig. 5). The cell numbers on the bottoms of the polystyrene dishes containing the tested materials ranged from  $19\,470 \pm 2\,200$  to  $316\,120 \pm 17\,650$  cells/cm<sup>2</sup>, and had a similar trend as on the corresponding material samples. The lowest cell numbers were found in the dishes containing 6-carboxycellulose with 6.6 wt% of  $-COOH$  groups, and the highest numbers were found in the dishes containing 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups modified with chitosan (Fig. 5).

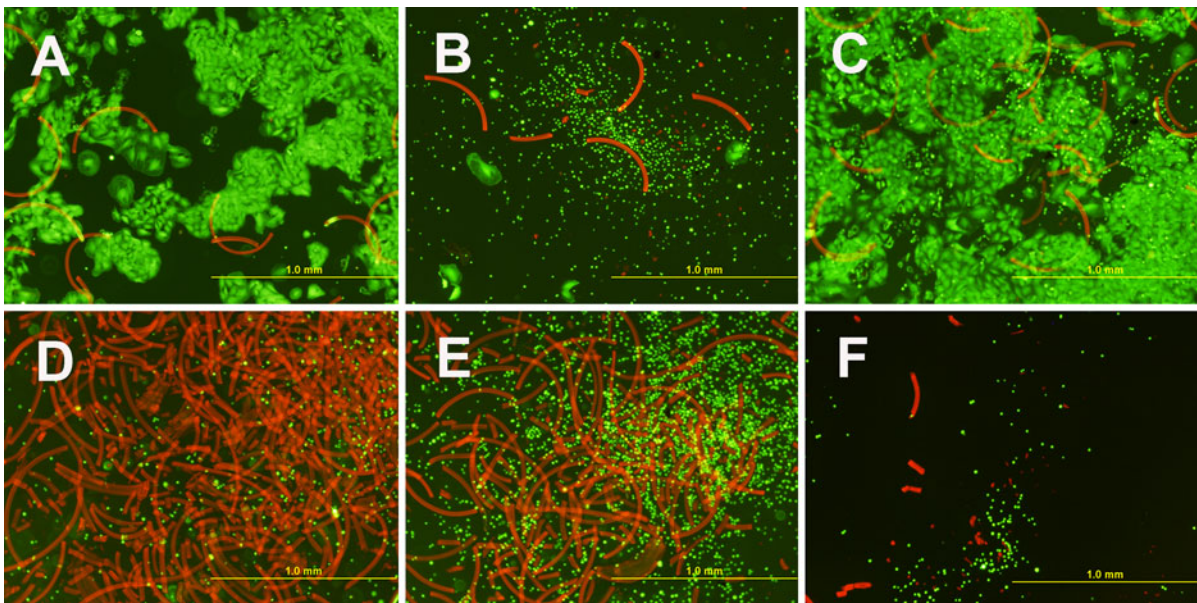
Positive effects of chitosan on the adhesion and growth of cells were also reported for vascular endothelial cells grown on cellulose acetate-chitosan

composites (Rubenstein et al. 2010), skin keratinocytes on bacterial cellulose–chitosan films (Kingkaew et al. 2010) or for 3T3 fibroblasts on cellulose sulfates and chitosan sulfates (Weltrowski et al. 2012). Chitosan has also been shown to have positive influence on cell differentiation, e.g. on the osteogenic differentiation of C2C12 myoblasts in combination with the bone morphogenetic protein-2 (Peschel et al. 2012). In accordance with this, functionalization of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups with chitosan in our study significantly increased the concentration of alpha actin and SM1 and SM2 myosins, i.e. markers of VSMC differentiation toward the contractile phenotype, in cells on these samples (see below). The positive effects of chitosan on the cell performance on cellulose-based materials have been explained by several factors, e.g. improvement of the mechanical properties of the cellulose materials by chitosan (Rubenstein et al. 2010), protection of growth factors against their proteolytic cleavage by chitosan (Weltrowski et al. 2012), and interaction of positively charged amino groups of chitosan and negatively charged cell membranes (Kingkaew et al. 2010). In this context, derivatives of microdispersed oxidized cellulose and arginine, which also contains positively charged amino groups, stimulated the proliferation of human peripheral blood leukocytes and mouse splenocytes (Jelinkova et al. 2002). Positively charged



**Fig. 6** Morphology and viability of vascular smooth muscle cells on day 7 after seeding on oxidized cellulose with 2.1 wt% of  $-COOH$  groups (**a, b, c**) and on oxidized cellulose with 6.6 wt% of  $-COOH$  groups (**d, e, f**); materials with no

modification (**a, d**), materials functionalized with arginine (**b, e**), materials functionalized with chitosan (**c, f**). LIVE/DEAD staining, obj. 4 $\times$ . Olympus IX 51 microscope, DP 70 digital camera, obj. 4 $\times$ , bar = 1 mm



**Fig. 7** Morphology and viability of vascular smooth muscle cells on day 7 after seeding on the bottoms of polystyrene dishes containing oxidized cellulose with 2.1 wt% of  $-COOH$  groups (**a, b, c**) and on oxidized cellulose with 6.6 wt% of  $-COOH$

groups (**d, e, f**); materials with no modification (**a, d**), materials functionalized with arginine (**b, e**), materials functionalized with chitosan (**c, f**). LIVE/DEAD staining, obj. 4 $\times$ . Olympus IX 51 microscope, DP 70 digital camera, obj. 4 $\times$ , bar = 1 mm

materials are also believed to improve the adsorption of cell adhesion-mediating extracellular matrix (ECM) proteins, such as fibronectin, vitronectin,

collagen and laminin from biological fluids to materials in an advantageous geometrical conformation, which promotes binding between cell adhesion

receptors and their ligands in the ECM molecules (for a review, see Bacakova et al. 2011). Interestingly, the positive effects of chitosan functionalization were seen not only in cells growing directly on the tested materials, but also in cells on the underlying PS dishes. In the case of 6-carboxycellulose with 2.1 wt% of  $-COOH$ , the increase in cell number was even more apparent on the underlying PS dishes than directly on the materials. Conversely, in the case of materials less appropriate for cell adhesion and growth, such as 6-carboxycellulose with 6.6 wt% of  $-COOH$ , the cell number was also low on the underlying PS dishes (Fig. 5). This suggests that our materials may release factors influencing cell behavior into the cell culture medium. In case of 6-carboxycellulose with 6.6 wt% of  $-COOH$ , the high acidity of this material probably also had a negative influence on the adhesion and growth of cells on the materials and on the underlying PS dishes.

Higher oxidation of 6-carboxycellulose caused not only higher acidity of this material, but also lower stability of the material in the cell culture environment. The content of 6.6 wt% of  $-COOH$  groups appeared to be too high from this point of view, as these samples disintegrated in the culture medium when they were subjected to simple manipulation. The stability of 6-carboxycellulose with 2.1 wt% of  $-COOH$  was sufficiently high, as it did not disintegrate when the samples were manipulated, and its degradation in the cell culture system was relatively slow in comparison with the samples with 6.6 wt% of  $-COOH$ .

#### Morphology and viability of VSMC on cellulose-based materials

There was also an observable contrast in the cell morphology and viability of the materials. On the 6-carboxycellulose samples with 2.1 wt% of  $-COOH$  groups (unmodified or modified with arginine, and particularly with chitosan), the cells were relatively well-spread, as indicated by their spindle-like shape, elongated in the direction of the material fibers (Fig. 6). By contrast, the cells on the 6-carboxycellulose with 6.6 wt% of  $-COOH$  groups were spherical, which indicates weak adhesion and spreading of these cells. Nevertheless, staining with the LIVE/DEAD viability/cytotoxicity kit revealed high viability of the cells. Most cells were stained with green fluorescence,

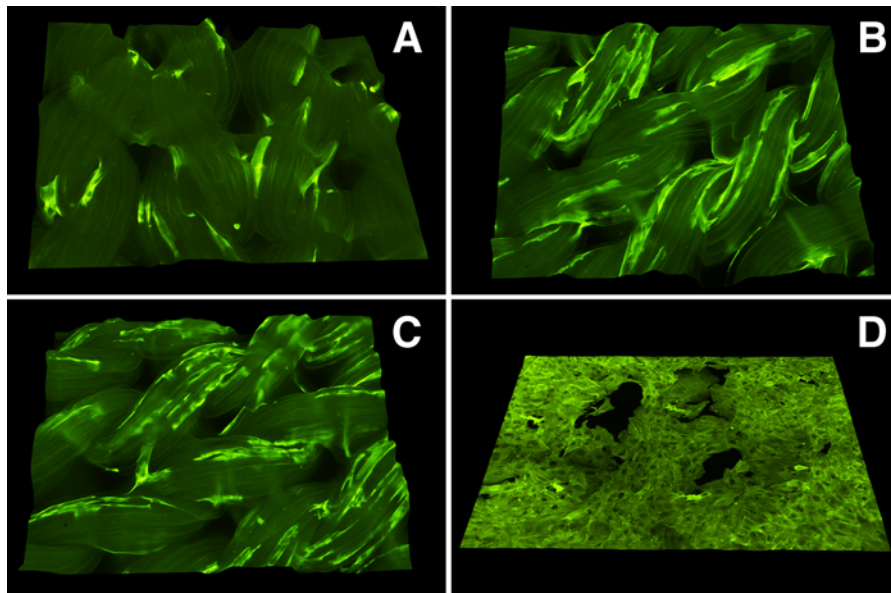
which indicates esterase activity in these cells. Only a few cells were stained in red, and were thus dead, even on the materials less appropriate for cell adhesion. Interestingly, some material fibers were non-specifically stained in red, or emitted a red autofluorescence (e.g., Fig. 6d).

A similar picture was found in cells grown on polystyrene dishes in the presence of cellulose-based materials. The cells in the presence of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups (unmodified, and modified with chitosan) were mostly well-spread, polygonal and morphologically similar to the cells on the control polystyrene dishes without materials. In the presence of the other materials, the cells were round and non-spread, although they are mostly viable. The fibrous structures (stained in red) which remained on the bottom of the polystyrene dishes after the material samples were removed, indicate degradation of the materials, particularly 6-carboxycellulose with 6.6 wt% of  $-COOH$  in unmodified state (Fig. 7d).

#### Phenotypic maturation of VSMC on cellulose-based materials

In a mature healthy blood vessel, the vascular smooth muscle cells are in so-called contractile phenotype. This phenotype is characterized by the presence of contractile proteins  $\alpha$ -actin and SM1 and SM2 isoforms of myosin, muscle type of tropomyosin, T-troponin, h-caldesmon, h1-calponin, meta-vinculin, desmin—a protein of intermediate filaments, and other specific markers. The migratory and proliferative activity of contractile VSMC is very low or none, and the amount of organelles involved in proteosynthesis, such as endoplasmic reticulum, ribosomes or Golgi complex, is also low. However, the synthetic phenotype of VSMC is manifested by high migration and proliferation activity of VSMC, loss of  $\alpha$ -actin and SM1 and SM2 myosins, predominance of  $\beta$ - and  $\gamma$ -isoforms of actin and non-muscle myosin, and an increased amount of synthetic organelles. This phenotype occurs physiologically in immature blood vessels under development, pathologically in diseased blood vessels, e.g. during atherosclerosis, hypertension and inflammation, and artificially after disintegration of the vascular wall and seeding VSMC in vitro (for a review, see Orr et al. 2009; Beamish et al. 2010; Parizek et al. 2011).





**Fig. 8** 3D reconstruction of the surfaces of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups without functionalization (a), functionalized with arginine (b) or chitosan (c), and of a control microscopic glass coverslip (d), colonized with rat aortic smooth muscle cells (day 7 after seeding). The cells were

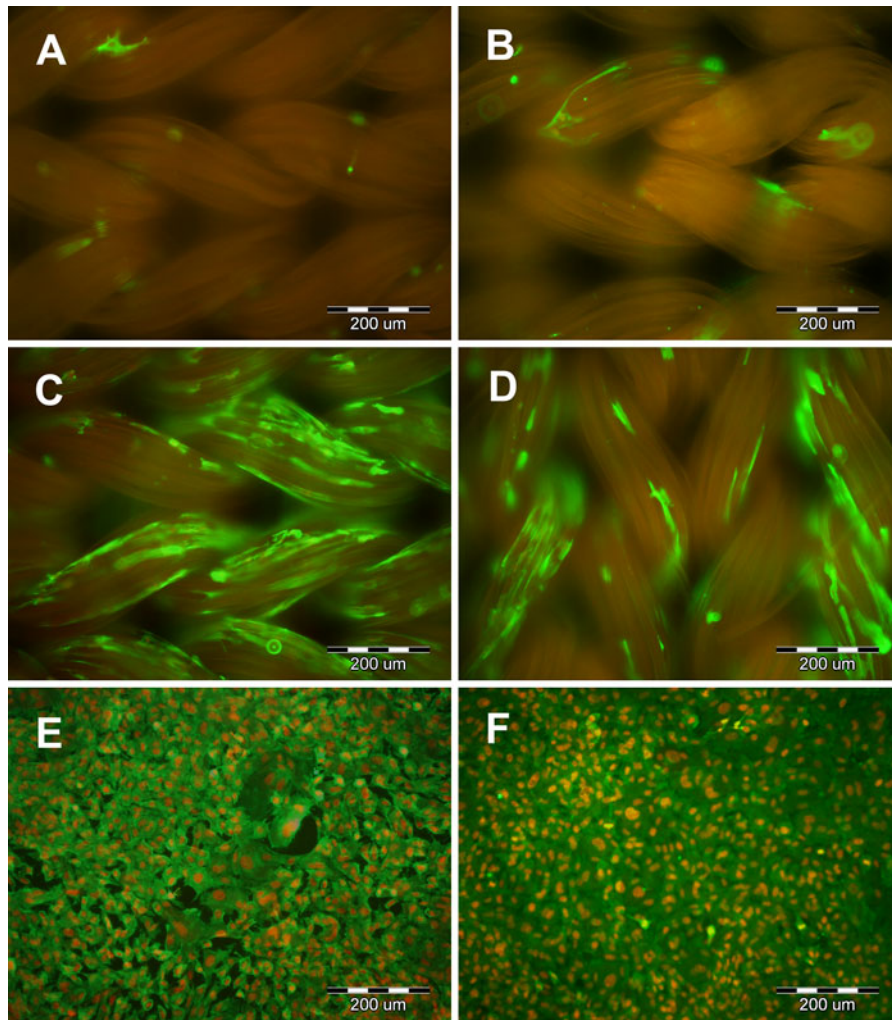
immunostained against alpha-actin. Nikon Eclipse Ti-E microscope, obj.  $10 \times 1.5$ ; Nikon DS-Qi1 MC digital camera, NIS-Elements AR software, version 3.10. Summarization of Z-sections with deconvolution

However, the contractile phenotype of VSMC *in vitro* can be restored, at least partially, by the culture conditions, e.g. by appropriate dynamic cultivation, composition of cell culture media and also by the physical and chemical properties of the growth substrate. The maturation of VSMC towards the contractile phenotype was most pronounced on materials based on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups. Immunofluorescence showed that the cells on these materials, and also on the underlying polystyrene dishes, were brightly stained for  $\alpha$ -actin and SM1 and SM2 myosins, particularly the cells on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups modified with chitosan. However, the actin and myosin fibers in these cells were less distinguishable, because the cells on these materials were markedly elongated along the cellulose fibers, and thus very thin (Figs. 8, 9). On the control flat surfaces, namely polystyrene dishes and microscopic glass coverslips, the cells were polygonal and were filled with  $\alpha$ -actin- or myosin-containing filaments, distinguishable at higher magnifications (data not shown here).

ELISA confirmed that after functionalization of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups

with chitosan or arginine, the concentration of  $\alpha$ -actin in the cells on these samples increased significantly in comparison with all other materials, including the control polystyrene dishes. The concentrations of SM1 and SM2 myosins in cells on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups with chitosan or arginine or in its unmodified state also became significantly higher than in cells on 6-carboxycellulose with 6.6 wt% of  $-COOH$  groups and the control polystyrene (Fig. 10).

The tested materials based on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups allowed considerable adhesion and spreading of VSMC (while on the 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups samples, the cells remained rather round). However, the fibers in the knitted cellulose fabric, aligned in parallel, guided the shape of the adhering VSMC to be elongated, i.e. reminiscent of the physiological spindle-like shape of contractile and quiescent VSMC in the tunica media of mature healthy blood vessels (Villaschi et al. 1994). In some *in vitro* studies, elongated and spindle-like shape of the VSMC was also associated with lower proliferation activity and a higher differentiation status of these cells (Chen et al. 2011). Another factor supporting the phenotypic



**Fig. 9** Immunofluorescence staining of alpha-actin (**a, c, e**) and SM1 and SM2 myosins (**b, d, f**) in cells growing on 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups (**a, b**), 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups modified

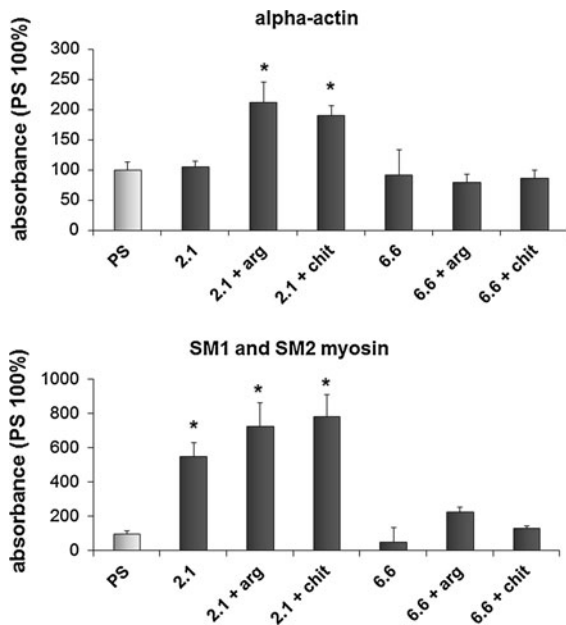
with chitosan (**c, d**) and on control polystyrene dishes (**e, f**). Day 7 after seeding, Olympus IX 71 microscope, DP 70 digital camera, obj. 20 $\times$ , bar = 200  $\mu m$

maturation of cells could be the acidity of the oxidized cellulose materials, which in the case of 6-carboxycellulose with a low concentration (2.1 wt%) of  $-COOH$  groups was relatively mild, and did not kill the cells but only restricted their proliferation and thus started their differentiation program.

Thus, 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups would be suitable for tissue engineering applications where differentiation and phenotypic maturation of cells is required rather than their high proliferation rate. An example of an application of this kind is the construction of bioartificial vascular

replacements, in which an excessive proliferation of VSMC can cause stenosis or obliteration of their lumen (Mol et al. 2008; Shinoka and Breuer 2008). Another positive factor is the relatively low degradability and high stability of 6-carboxycellulose with 2.1 wt% of  $-COOH$  groups. In other degradable polymers considered for the construction of vascular replacements, the degradation time is usually too short for the creation of new tissue, which can lead to the development of an aneurysm (Greisler et al. 1991). For example, polylactides, polyglycolides and their copolymers usually degrade within weeks and months,





**Fig. 10** Concentration of alpha-actin and SM1 and SM2 myosin in VSMC on day 7 after seeding on 6-carboxycellulose materials (see legend to Fig. 5). Measured by ELISA per mg of protein, absorbance values were expressed in % of values obtained from cells grown on control PS. Mean  $\pm$  S.E.M. from 6 measurements from 2 independent samples for each experimental group. ANOVA, Student–Newman–Keuls method. Statistical significance ( $p \leq 0.05$ ): alpha-actin: *asterisk* values in 2.1+chit and 2.1+arg are significantly higher than on all other samples; Myosin: *asterisk* Values in 2.1, 2.1+chit and 2.1+arg are significantly higher than in all other samples

whereas for human vessel reconstruction, the degradation time should be considered to be more than 1 year, particularly in older and polymorbid patients with very slow tissue regeneration.

In order to further improve the attractiveness of cellulose-based materials for cell colonization, not only modification with chitosan and arginine but also other modifications can be considered. These modifications involve plasma, ion or UV irradiation, which are proved to increase the attractiveness of synthetic polymers for cell adhesion and growth (Parizek et al. 2009; for a review, see Bacakova et al. 2011). Plasma modification increased the adsorption of chitosan to cellulose (Fras Zemljic et al. 2009). Another important modification is functionalization with various biomolecules, such as fibronectin (Ko and Iwata 2001), and particularly ECM-derived adhesion oligopeptides (e.g., RGD), which has also been successfully applied in cellulose-based materials (Bodin et al. 2007).

## Conclusion

The results that we have obtained indicate that oxidized cellulose with 2.1 wt% of  $-COOH$  groups could be an appropriate material for tissue engineering, due to its relatively high stability during manipulation and exposure to the cell culture environment, and its biocompatibility, which can be further improved by modification with biomolecules, particularly chitosan. As it induces the most appropriate spreading and phenotypic maturation of cells, but not considerable cell proliferation, it could be used in the construction of bioartificial tissues or organs, where high proliferation activity of cells is not desired. A possible application of this material is for constructing bioartificial vascular prostheses, where excessive growth activity of cells on the material may cause stenosis and failure of these replacements.

**Acknowledgments** This study was supported by Grant No. 2A-1TP1/073 of the Ministry of Industry and Trade of the Czech Republic, and by Grant No. P108/12/1168 of the Grant Agency of the Czech Republic. Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for his language revision of the manuscript.

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