SURFACE CHARACTERIZATION OF CHITOSAN FILMS PHOTOCHEMICALLY MODIFIED BY RGDS AND EGF

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Abstract: In the presented study, ionically crosslinked membranes were modified by immobilizing RGDS (Arg-Gly-Asp-Ser) sequence of cell adhesion factor, fibronectin, or cell growth factor EGF (epidermal growth factor) for the possible use in tissue engineering applications. The immobilization was realized by using photochemical techniques, in which the biosignal molecules were first reacted with a heterobifunctional crosslinker sulfo-SANPAH (sulfosuccinimidyl 6 (4'-azido-2'-nitrophenyl-amino) hexanoate), to gain photoreactive properties. The photoreactive biosignal molecules were then immobilized on the chitosan membrane by UV irradiation. The effectiveness of immobilization process was checked with ATR-FTIR and XPS analysis. While the ATR-FTIR results showed the successful immobilization, the XPS studies revealed the increase in amount of nitrogen and O/N and C/N ratios decreased for both RGDS and EGF modified membranes. The results obtained from the AFM studies to investigate the surface morphology showed that the immobilization procedure has no significant effect on surface roughness.

Introduction

Chitosan is a polycationic biopolymer obtained by alkaline deacetylation of chitin, which is the main component of exoskeleton of crustaceans, insects and the cell walls of fungi [1,2]. In recent years, chitosan is receiving a great deal of interest for tissue engineering applications as a material derived from nature[3]. Besides its low cost and ecologically interesting production chitosan is known for being biocompatible allowing its use in implantable or injectable systems. As its biological, physical and chemical properties can be controlled and engineered under mild conditions, chitosan has been the appealing choice for various applications such as drug delivery systems, orthopaedic/periodontal composites, scaffolds for cell culture and wound-healing management . Through these applications chitosan-based materials are prepared in the form of powders and flakes but foremost as hydrogels. One of the methods to prepare chitosan hydrogels is to use crosslinkers to interconnect the polymer chains leading to the formation of a 3D network. Recently much attention has been focused on ionic crosslinkers for the preparation of chitosan hydrogels with keeping its biocompatible properties.

Besides its comprehensive properties as a biopolymer, additional approaches have been investigated to improve the properties of chitosan as a biomaterial in tissue engineering applications. Biomaterial modification by immobilization of various cell recognition motifs and growth factors to obtain controlled interaction of cells and induced tissue growth has been the major strategy in developing engineered tissue substitutes. Among these biosignal molecules, arginine-glycine-aspartic acid (RGD) sequence of cell adhesion protein fibronectin, has been by far the most effective and most often employed peptide sequence for stimulated cell adhesion[4]. In recent years the tetrapeptide arginine-glycine-aspartic acid-serine (RGDS) is receiving much attention as one of the most effective cell adhesive moiety[5]. Additionally, growth factors are the most important signalling molecules which promote cell proliferation, migration and differentiation in all advanced organisms. Among the growth factors, epidermal growth factor (EGF) has fundamental functions throughout the organism. It has particular bioactivities besides being a proangiogenic biomolecule with its chemotactic properties on endothelial cells, such as stimulation of cell proliferation, differentiation of the epithelia and wound healing [6] .

In the presented study, ionically crosslinked chitosan hydrogels were prepared in the membrane form and modified by the cell recognition motif RGDS (arginine-glycine-aspartic acid-serine) and cell growth factor EGF to provide a bioactive natural material for tissue engineering applications. The immobilization of biological molecules was realized by using photochemical immobilization techniques based on phenyl azido chemistry [7,8]. The biological molecules were first attached to water-soluble functional moiety to form phenyl-azido derivatized proteins or peptides and then they were grafted to material substrates by ultraviolet (UV) irradiation. RGDS and EGF were immobilized on ionically crosslinked chitosan hydrogels by inducing the reactions between azido group and hydroxyl group of the chitosan structure. Fourier

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transform infrared spectrometer-attenuated total reflectance (FTIR-ATR) spectra for functional group analysis and X-ray photoelectron spectroscopy (XPS) analysis were performed in detail to characterize the chitosan, crosslinked chitosan and RGDS and EGF modified chitosan membranes. Atomic force microscopy (AFM) was used to investigate the surface morphology.

Materials and Methods

Chitosan was purchased from Sigma (Germany) (85% of deacetylation). Cell adhesion factor arginine-glycine-aspartic acid-serine (RGDS) and Epidermal Growth Factor (EGF) were both obtained from Sigma (Germany). Photochemical cross-linker sulfosuccinimidyl 6 (4'-azido-2'-nitrophenyl-amino) hexanoate (sulfo-SANPAH) was purchased from Pierce (U.S.A). Acetic acid (glacial) and sulphuric acid (98%) were obtained from Merck (Germany). Phosphate Buffer Saline (PBS) tablets were obtained from Sigma (Germany).

In order to prepare the crosslinked chitosan membranes, 1.0 g of chitosan was first dissolved in 100 ml of 1% (v/v) acetic acid solution, poured into glass Petri dishes and air dried. After the membranes were detached from the glass surface, they were submerged into a crosslinking solution comprising 0.02 M sulfuric acid in 50 % (v/v) aqueous acetone solution for 1h [34, 35]. The crosslinked chitosan membranes (with a thickness of ~200 μm) were washed with deionized water and air dried.

The crosslinked chitosan membranes were modified by the RGDS sequence of cell-adhesion protein, fibronectin or cell growth factor EGF. The modification of chitosan with RGDS was realized by using the photochemical fixation method [32]. First RGDS (2 mM) and the heterobifunctional crosslinker sulfo-SANPAH (2 mM) were dissolved in PBS (pH: 7.4) separately. Then equivalent moles of the two solutions were gently mixed and allowed to react in dark at room temperature for 2 h. The resulting solution of phenyl-azido derivatized photo-reactive peptides was poured on the chitosan membrane and allowed to dry at room temperature. To induce the photochemical fixation reaction ultraviolet light was applied on the chitosan membrane for 3 minutes by using a UV lamp (Osram, Germany). After that the membranes were fully rinsed with deionized water to remove the unreacted reagents. EGF immobilization was also performed by using the same procedure. EGF was dissolved in PBS (0.01 mg/ml) and reacted with 2 mM, 1 ml sulfo-SANPAH (in PBS, pH:7.4) overnight in dark at 4°C. The resulting photo-reactive EGF solution was poured on the chitosan membranes, air dried and UV irradiated with the same conditions for RGDS immobilization.

Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectra for the chitosan (without crosslink), crosslinked chitosan (unmodified), RGDS-modified and EGF-modified chitosan membranes were obtained by using a Perkin-Elmer Spectrum One IR spectrometer (USA). The spectra of samples were taken at 400-4000 cm^{-1} wavelength and analyzed with a standard software package, Perkin-Elmer, Spectrum One.

XPS analysis was performed by using a PHI 5600 Multi Technique Spectrometer equipped with dual Al/Mg anode, hemispherical analyser and electrostatic lens system (Omni Focus III). The electron take-off angle was typically 45°, corresponding to a sampling depth of about 6 nm. The analyser was operated in FAT mode by using the Al $K\alpha_{1,2}$ radiation with pass energies of 187.5 eV for survey scans and 11.75 eV for the detailed scans. The pressure in the main vacuum chamber during the analysis was kept below $3x10^{-8}$mbar. The binding energy of 285.0 eV of the main C1s component (assigned to C-C and C-H bondings) was used as a reference to calibrate the energy position of the various peaks. The photoelectron peaks were analysed by using a curve-fitting routine based onto Gaussian shapes and a linear Shirley background subtraction approximation.

The surface roughness and morphology at micro- and nanometer scale were measured with a Multimode/Nanoscope IIIA Atomic Force Microscope (VEECO, USA) in tapping mode in air with a standard silicon tip. The relative room humidity was 30 % and the room temperature was 23°C. Data were acquired on square frames having edges of 5 μm, 1 μm and 500 nm. Images were recorded using both height and phase-shift channels with 512x512 measurement points (pixels). Measurements were made twice or three times on different zone of each sample. Surface roughness values were determined in three random areas per sample, scanning across areas 1x1 μm^2. Roughness parameters (R_a and R_{ms}) calculation and image processing were performed using the Nanoscope III software.

Results and Discussion

Crosslinked chitosan membranes were prepared by using sulphuric acid as crosslinker. Divalent sulphate ions crosslink the chitosan main chains ionically, by exploiting the Coulombic interactions with the protonated amino groups on adjacent chitosan chains. The effect of the crosslinking process has been studied in detailed by means of FTIR-ATR spectroscopy for relatively thick layers (1-2 μm) and XPS for the shallowest layers (about 10 nm thick) at the membrane surface. Faint absorption around 1550 cm^{-1} due to NH_2 deformations (amide I) and absorption around 1640 cm^{-1} for C=O bond (amide II) shift to 1528 cm^{-1} for amide I and 1630 cm^{-1} for amide II band after the crosslinking process, in agreement with previous reports on crosslinking effects in chitin/chitosan-based materials. XPS data reveal that the surface composition of crosslinked chitosan does not change significantly with respect to uncrosslinked chitosan membranes and the only evidence is a certain amount of sulphur, coming from the crosslinker (Table 1). In the FTIR-

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ATR spectra for RGDS and EGF modified chitosan membranes the peak associated with the azide group in sulfo-SANPAH at 2120 cm^{-1} are clearly observed for both RGDS and EGF modified membranes. The broad shoulders at approximately 1566 cm^{-1} and 1414 cm^{-1} are due to the amide II regions and carboxyl groups, respectively which are both present in RGDS and EGF (Fig. 1).

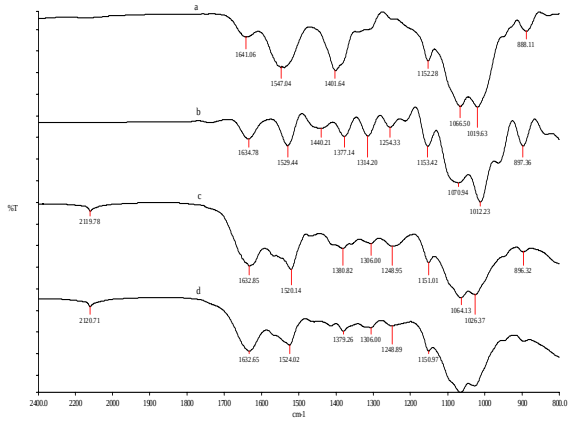

Figure 1: FTIR-ATR spectra for (a) uncrosslinked, (b) crosslinked, (c)RGDS-modified, (d) EGF-modified membranes.

The XPS data suggest that RGDS and EGF were successfully immobilised on crosslinked chitosan membranes in fair agreement with the FTIR-ATR data. The N 1s peak shapes and surface atomic composition values indicate that the peptide-related component at 399.9±0.2 eV is strongly increased with respect to the ammonium groups at 401.8±0.2 eV after immobilization of RGDS and EGF (Table 1).

Table 1: Average surface atomic composition for chitosan membranes.

	O 1s (at.%)	C 1s (at.%)	N 1s (at.%)
Uncrossinked chitosan	27.2	67.0	5.8
Crosslinked chitosan	29.6	63.0	5.4
RGDSmodifiedchitosan	24.0	69.8	6.2
EGF-modified chitosan	24.3	66.3	8.7

Fig. 2 and Fig. 3 show the AFM images of uncrosslinked and crosslinked, while Fig. 4 and Fig 5. report RGDS- and EGF-modified chitosan membranes. Table 2 reports the quantitative data on roughness in terms of root mean squared (R_{ms}) and averaged (R_a) roughness.

Table 2: R_{ms} and R_a values on 1 µm scale for various chitosan surfaces

	R_{ms} (nm)	R_a (nm)
uncrossinked chitosan	5.11±1.10	3.41±0.69
crosslinked chitosan	2.16±0.16	1.71±0.13
RGDS-modified chitosan	2.60±0.44	1.93±0.32
EGF-modified chitosan	1.95±0.15	1.50±0.15

Figs. 2 and 3 show that the crosslinking procedure strongly modify the surface morphology of chitosan. In fact, the unmodified chitosan exhibits a quite dense coverage by features of about 24±2 nm of height and typical diameters of 20-30 nm. After crosslinking, the surface topography becomes more homogeneous (see phase contrast image in Fig. 2b) and relatively flat (see Table 2), with smooth features of regular average diameter, about 15-20 nm. This effect could be explained in terms of the formation of bundles of chitosan fibers, due to the crosslinking process, while the highly inhomogeneous topography before crosslinking could be due to uneven termination of free fibres.

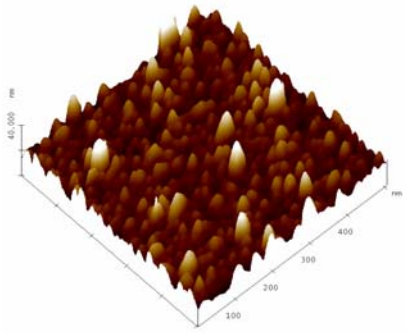

Figure 2: AFM of uncrosslinked chitosan.

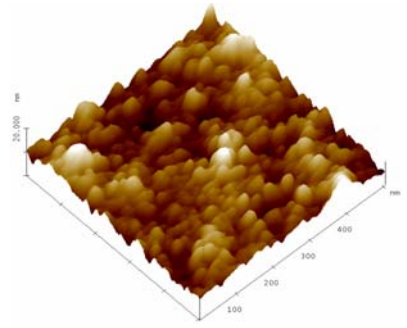

Figure 3: AFM of crosslinked chitosan.

As to the chitosan modified with RGDS (Fig. 4), the AFM shows the formation of relatively large

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aggregates, which apparently follow the structure of the underlying substrate. In this case the roughness is slightly higher than the one of the uncovered surfaces. On the other hand, in the case of EGF-modified samples (Fig. 5), the surface morphology results smoothed and it seems basically formed by nanometric-sized fibril-like structures, homogeneously covering the substrate. The roughness is lower than the one of the uncovered substrates.

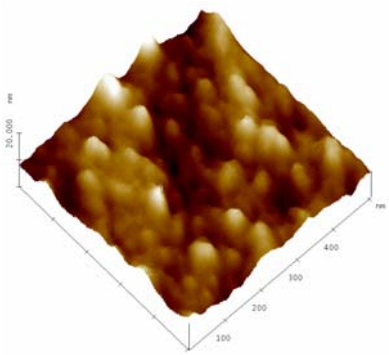

Figure 4: AFM of RGDS-modified chitosan.

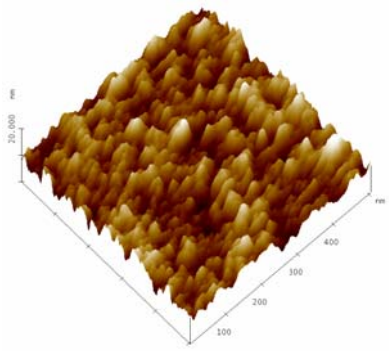

Figure 5: AFM of EGF-modified chitosan.

Conclusion

Chitosan membranes were successfully modified with RGDS or EGF by using photochemical immobilization techniques for facilitating its applications in tissue engineering. The results obtained from characterization studies including FTIR-ATR, AFM and detailed XPS measurements revealed that photochemical immobilization technique is a powerful method for immobilizing biological molecules without changing the surface roughness of the chitosan membranes. For the future perspectives, in our ongoing studies, this technique will be applied for the micropatterning of the chitosan membranes with biosignal molecules. This strategy will be utilized to place the cells in specific locations for controlled orientation to create organized structures and precisely defined cellular architectures.

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