JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 10, No. 4, April 2008, p. 933 - 936

Type I collagen-TiO_2 aerogel based biocomposites

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Composites based on type I collagen and TiO_2 have been prepared by liophylisation. Structural and morphological analyses have been performed by means of FT-Raman, FT-IR and porosimetry measurements in order to find the influence of the TiO_2 on the collagen structure. The structural information derived from the Raman spectra analysis clearly proof the existence of titania structural units inside the porous composites, but only for the highest TiO_2 content (12 wt%). The average pores diameter and the pores density of the lyophilised samples were determined and a relationship with the titania content has been found. The fingerprint features of all FT-IR spectra are specific to the type I collagen and their close analysis revealed conformational changes of the collagen proteins caused by the TiO_2 addition, however, the integrity of the triple helicity was found to be unaltered. *In vitro* tests performed on fibroblast (FB) and osteoblast (OB) cells culture have revealed a very good biocompatibility of the composites, especially in the case of the 12 wt% TiO_2 containing sample.

(Received November 13, 2007; accepted April 2, 2008)

Keywords: Biocomposites, Collagen, TiO_2, Raman, IR, Biocompatibility

1. Introduction

Collagen is one of the omnipresent proteins in the vertebrates' body. It occurs in several polymorphic forms, of which the most common type is type I, one of the constituents of animal and human skin, bone, tendon, teeth, nail, and cell walls. Type I collagen exists as a triple-stranded helix, containing two identical polypeptide chains and a third chain, which has a different amino acid sequence [1]. Each chain forms a left-handed helix with three residues in each turn. The three left-handed helices are twisted together to the right to give a coiled coil. This suprahelical structure is stabilized by numerous interstrand hydrogen bonds conferring remarkable mechanical properties to this protein [2].

Collagen molecules are associated into fibrils, which are grouped into larger fibres that make up the extracellular matrix of the connective tissue [1]. Collagen is a natural material with good biocompatibility and a well-characterized low antigenicity. It can be degraded into well-tolerated physiological compounds. Therefore, collagen has attracted great interest as a biomaterial in medical use, such as prosthesis, drug carrier, tissue engineering and so on [3, 4].

Relative recently [5] the shielding effect of TiO_2 nanoparticles on collagen under ultraviolet irradiation has been investigated by using UV spectrophotometry. It was found [5] that TiO_2 nanoparticles, in quite low content (0.5 wt% and 2.5 wt%), have a photochemical stabilizing effect on the collagen, the higher the TiO_2 amounts in collagen, the greater the shielding effect of UV radiation.

In the present work, our aim was to synthesize collagen-TiO_2 aerogels based composites with a relatively high TiO_2 content and to find out the structural and morphological influence of relatively high titania amount on the collagen proteins. Our further interest was focused on *in vitro* testing the as-prepared composites by using fibroblast and osteoblast cells culture.

2. Experimental

The collagen matrix was obtained from type I collagen gels, extracted from bovine hides dermis by chemical and enzymatic treatments performed in alkaline and acid media at temperatures lower than 25°C [6]. The TiO_2 gels were obtained by sol-gel method using Ti (IV) isopropoxide, anhydrous ethanol, deionised H_2O and HNO_3 (molar ratio 1:20:3:0.08). The gels were supercritical dried (T > 35°C, p > 1200 psi) by using a SAMDRI-PVT 3D (Tousimis) critical point dryer and liquid CO_2 and TiO_2 aerogels were obtained [7]. The type I collagen gel was mixed with the TiO_2 aerogels (3-15 wt%) and the obtained biomaterial was dried by freezing and sublimation. The TiO_2 concentrations in the as obtained samples were found to be 3.5 wt% and 12 wt% by inductively coupled plasma atomic emission spectrometry.

The average pores diameter and their density were determined by employing a COULTER Porometer II device.

♣ paper presented at Symposium on Biomaterials, Cluj-Napoca, Romania, October 18-20, 2007

The FT-Raman and FT-IR spectra were recorded with a Bruker Equinox 55 spectrometer with an integrated FRA 106 Raman module. A radiation of 1064 nm from a Nd-YAG laser was employed for the recording of the Raman spectra. The Raman measurements were performed by using a power of 50 mW incident on sample and a spectral resolution of 2 cm^{-1}. In the case of IR measurements the spectral resolution was also of 2 cm^{-1}.

In vivo tests of the collagen matrix and composite samples were performed by using fibroblast (FB) and osteoblast (OB) cells culture. The collagen matrix and the cells culture without substrate were used as reference. The samples were firstly sterilized and further the FB and OB cells originating from the cell lines CCD 1070 and G 292 were grown up. The cells density was of $3.5x10^5$ cells/plate. The cells culture were incubated at 37^0C into wet atmosphere with 5% CO_2 content and were monitored from the cytomorphology point of view at 24 and 48 h from incubation. The samples were analyzed with a phase contrast microscope Nikon TS 100, the pictures being recorded with a Nikon Cooplix 4500 digital camera.

3. Results and discussion

The Raman spectra of the as-prepared samples are displayed in Fig. 1 (b-d). The well- known amide I band, which is almost entirely due to the carbonyl stretching vibration, together with the complex amide III band, which is due to the CN stretching and NH in plane bending from amide linkages and also to the wagging vibrations of he CH_2 groups, appear in the collagen spectrum in the 1615 - 1715 and 1240 - 1272 cm^{-1} spectral range. These bands can be also observed in both composites spectra. With the increase of the TiO_2 content a few spectral changes occur.

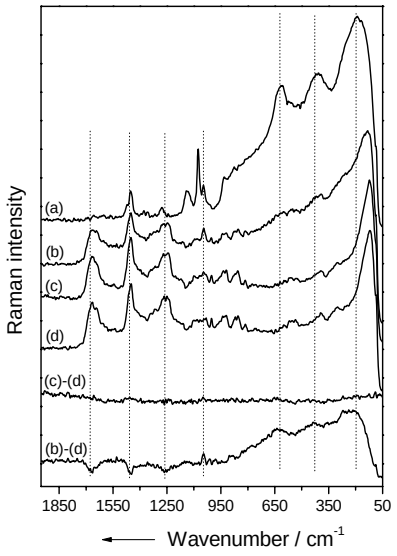

Fig. 1. Raman spectra of TiO_2 aerogel sample (a), collagen-TiO_2 aerogel composite with 12 wt% TiO_2(b), collagen-TiO_2 aerogel composite with 3.5 wt% TiO_2 (c), collagen matrix (d), together with two difference spectra as indicated.

In order to clearly evidence these spectral differences and consequently to evaluate the structural modifications the subtraction of the collagen matrix spectrum from the composites spectra was independently performed and the resulted difference spectra are presented in Fig. 1 (c)-(d) and (b)-(d). The addition of 3.5 wt% TiO_2 to the collagen matrix does not induce any Raman sensitive change (Fig. 1(c)-(d)). For a higher titania content (12 wt%) spectral changes can be seen in the difference Raman spectrum both in the small wavenumbers domain (100 - 800 cm^{-1}) as well as in the high wavenumbers region (800 - 1800 cm^{-1}) (Fig. 1(b)-(d)). The bands whose intensity increase in the up-down direction, i.e. the bands around 1264, 1453 and 1670 cm^{-1}, can be associated with the existence in the composite structure of a decreased number of groups similar with those that build up the collagen structure. On the other hand, the bands whose intensity increase in the down–up direction, i.e. the bands around 200, 430, 622 and 1045 cm^{-1}, can be associated with the existence in the composite structure of an increased number of the structural units that build up the TiO_2 aerogel structure. By applying such a judgment one can deduce that once a considerable amount of TiO_2 aerogel is added to the collagen matrix a diminishing of the amide III, amide I and CH_2 groups analogous with those from the collagen matrix occurs, whereas an increasing of the number of TiO_6 structural units (200, 430 and 622 cm^{-1}) [8] and other organic residues groups from the TiO_2 aerogel (around 1045 cm^{-1}) [9] takes place. Their presence is confirmed by the spectral features evidenced in the Raman spectrum of the pure TiO_2 aerogel (Fig. 1a). The existence of the above-mentioned Raman spectral changes could be a consequence of some significant morphological and structural modifications of the collagen proteins induced by the TiO_2 aerogel presence. Porosimetry measurements shown that both the pure collagen matrix and the composite samples have a sponge-like structure with highly polydispersed pores. As one can see from Table 1, the increasing of the TiO_2 content leads to the raising of the average pores diameter and consequently to the pores density decrease.

Table 1. The TiO_2 content from the investigated samples together with the average pores dimensions and their density.

TiO_2 weight %	$d_{average}$ [μm]	Pores density [pores / cm^2]
0-	2.05	7.89×10^9
3.5	2.94	7.20×10^4
12	10.46	3.77×10^3

In order to get further information about the structural modifications of the collagen caused by the TiO_2 aerogel presence inside the composites and particularly to ensure if the integrity of the triple helix structure is affected FT-IR spectra were recorded and are illustrated in Fig. 2.

As one can see all spectra show the spectral characteristics of type I collagen, which are denoted as amide I (1600-1740 cm^{-1}), amide II (1485-1590 cm^{-1}), amide III (1190-1300 cm^{-1}), amide A (around 3300 cm^{-1})

and B (around 3070 cm^{-1}) [10]. By looking at the FT-IR spectra one can remark their similarity fact that suggests that the collagen structure is not strongly affected by the TiO_2 addition. A close analysis of the specific spectral regions reveals some differences, especially between the spectrum of the sample with the highest TiO_2 content and that of the collagen matrix, that will be further discussed.

The amide I band, which exhibits a high sensitivity to conformational changes, is the frequently used spectral region for IR spectroscopic analysis of the secondary structure of proteins [11-13]. This band is centred in the range 1600-1740 cm^{-1} and presents a typical asymmetry due to the varying amounts of fine structure, including an absorption near 1632 cm^{-1} that could be related to hydrogen bonds involving carbonyls [13, 14], as can be seen in Fig. 2.

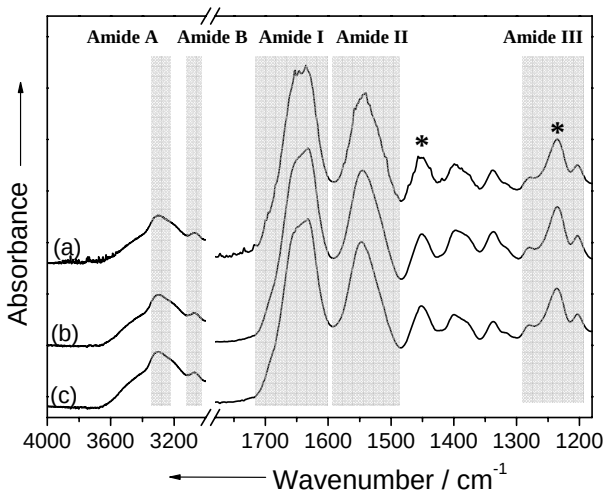

Fig. 2. FT-IR spectra of collagen-TiO_2 aerogel composite with 12 wt% TiO_2 (a), collagen-TiO_2 aerogel composite with 3.5 wt% TiO_2 (b) and collagen matrix (c)

Other well-defined absorption signals located at 1635, 1646 and 1654 cm^{-1} can be better observed in the spectrum of the sample with the highest TiO_2 content (see Fig. 2a). The band at 1635 cm^{-1} can be assigned to the β-sheet structure and the band at 1646 cm^{-1} to the unordered random coils, while the appearance of the peak at 1654 cm^{-1} indicates that the proteins adopt a α-helical configuration [15-18] The unordered component, usually referred to as random coil, has been defined in previous studies [12, 19] as a secondary structure of neither α-helix nor β-sheet. Its distinguishing feature is that it is nonrepetitive in the polypeptide backbone conformation [12]. The appearance of a peak at approximately the same position (1645 cm^{-1}) has been previously observed [13] during *in vitro* investigations of glutaraldehyde crosslinked type I collagen film under accelerated cyclic fatigue conditions. It was assumed that a change in the helicity occurred as a consequence of mechanically induced molecular fatigue and was emphasized that the presence of this new absorption signal is accompanied in all experimental events by the progressive appearance of new peaks at 1260, 1084 and 1015 cm^{-1}, which significantly increased in intensity with the duration of cyclic fatigue. The appearance of these features was associated with the collagen denaturation [13]. Any of these spectral changes do not appear in the IR spectra of the investigated composites. Thus, the distinguish appearance of the 1646 cm^{-1} band in the spectrum of the sample with 12 wt% TiO_2 could be related to a conformational change of the proteins secondary structure caused by the linkage of TiO_2 gel by collagen proteins. Other spectral changes between the spectrum of the collagen matrix and that of the sample with the highest TiO_2 content can be seen after a close inspection of the amide II band. This band is located between 1485 and 1590 cm^{-1} (see Fig. 2), its complex nature comprising vibrational sources in both NH deformation and CN stretching modes [14, 20]. As can be observed from Fig. 2 the addition of a significant TiO_2 amount (12 wt%) into the collagen matrix leads to a shift of the amide II band (1546 cm^{-1}), which is preponderantly given by the β-sheet structure [21], to lower wavenumber values (1540 cm^{-1}), while a small TiO_2 content does not produce any shift of this band at all. The collagen bands from this spectral region have been associated in the literature [14, 22] with the existence of the amide II mode in random coil (around 1535 cm^{-1}) and in helical form (around 1555 cm^{-1}). Thus, one can assume that the change of the peak position is due to a conformational modification of the proteins.

On the other hand, it was reported [23] that the integrity of the triple helix structure is attested when the absorbance ratio of the amide III band at 1235 cm^{-1} versus that at 1451 cm^{-1}, which is given by CH_3 bending vibrations, is higher than, or equal to, 1 (bands denoted in Fig. 2 with asterisk). IR studies carried out on gelatine membranes, which consist of highly denaturated collagen, show a ratio equal to 0.59, proving the loss of triple helicity [24].Other authors [23], who investigated the interaction of collagen with corilagin, have found a decrease of this ratio value to 0.8 on increasing the corilagin concentration as a result of the collagen triple helicity alteration. The intensities ratio values calculated from the spectra depicted in Fig. 2 were found to be 1.163, 1.222 and 1.146 for the collagen matrix, composite samples with 3.5 wt% and 12 wt% TiO_2, respectively. This result shows the integrity of the triple helicity even for the biocomposite sample containing relatively high TiO_2 content (12 wt%).

Having in view that in certain cases the amide A band was found to be more sensitive to structural changes in collagen than amide B, I and II bands [14] we further focused our attention on this spectral range. The amide A and B bands of collagen that are associated with the NH stretching modes appear at around 3300 and 3070 cm^{-1}, respectively [21]. It was pointed out [14] that the amide A band of collagen usually gives absorption signals at 3325-3330 cm^{-1}, but when the NH group of a peptide is involved in a hydrogen bond, its position is shifted to lower wavenumber values, at 3300 cm^{-1}. The presence of a maximum absorption signal at 3300 cm^{-1} in all recorded spectra (Fig. 2) indicates the involvement of the NH groups in hydrogen bonds. The similarities observed between the spectra of the collagen and biocomposite samples in this spectral range can be regarded as a proof of the insignificant conformational changes induced in the collagen structure by the TiO_2 addition. Once the supposition of collagen proteins degeneration was proved to be false our further interest was to test the potential of the composites as

biomaterials.

In vivo biological tests performed on the composite samples with FB and OB cells culture demonstrate that TiO_2 has a biostimulator effect on the cell metabolism, especially in the case of the osteoblasts. This effect was more pronounced when the composite samples with the highest titania content were tested.

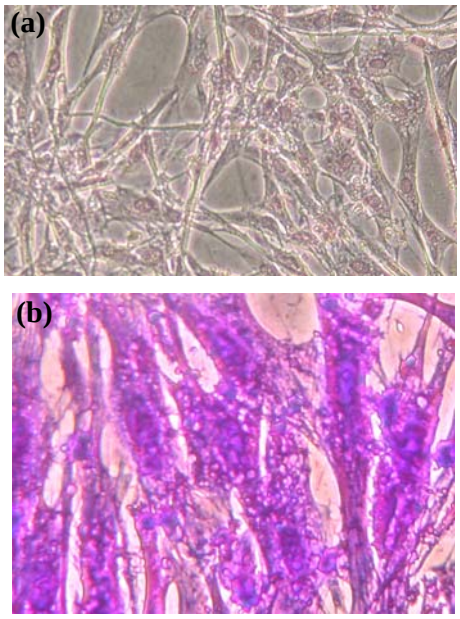

Fig. 3. FB (a) and OB (b) cells grown up on the composite sample with the 12 wt% TiO_2 content

Two of the most representative pictures illustrating the cells grown on the composite sample with 12 wt% TiO_2 are depicted in Fig. 3.

All these results give us confidence to perform supplementary investigations in order to evidence the yet not revealed structural characteristics of these biocomposites and especially to understand more about the structural particularities of the TiO_2-collagen linkages.

5. Conclusions

Composites based on collagen-TiO_2 aerogels with relatively high TiO_2 content (12 wt%) have been obtained by liophylisation process. The analysis of the Raman spectra revealed the presence of the titania structural units inside the composite sample with 12 wt % TiO_2. Porosimetry measurements showed that the biocomposites samples preserve the collagen sponge-like structure. It was found that as the TiO_2 content increases the average diameter of the pores increases and consequently the pores density decreases. The close examination of the IR spectra showed the existence of small conformational modification of collagen proteins structure, even for the highest TiO_2 content. The integrity of the triple helicity was found to be unaltered. The biostimulator effect on the cell metabolism, especially on that of the osteoblasts was proved by i*n vivo* tests performed on the composites.

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JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 10, No. 4, April 2008, p. 933 - 936