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Structure of collagen adsorbed on a model implant surface resolved by polarization modulation infrared reflection–absorption spectroscopy

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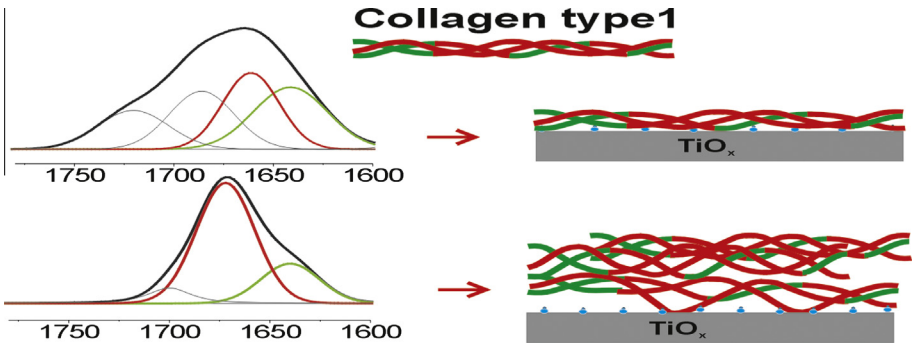
HIGHLIGHTS

- Amide I modes are assigned to structural units of the collagen triple helix.
- The triple helix of collagen adsorbed on the titania surface is distorted.
- Collagen interacts with water molecules adsorbed on the titania surface.
- Intramolecular hydrogen bonds at imino acid protein fragments are destabilized.
- Native collagen structure is restored upon addition of water to the protein film.

GRAPHICAL ABSTRACT



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ABSTRACT

The polarization modulation infrared reflection–absorption spectra of collagen adsorbed on a titania surface and quantum chemical calculations are used to describe components of the amide I mode to the protein structure at a sub-molecular level. In this study, imino acid rich and poor fragments, representing the entire collagen molecule, are taken into account. The amide I mode of the collagen triple helix is composed of three absorption bands which involve: (i) (~1690 cm^{-1}) the C=O stretching modes at unhydrated groups, (ii) (1655–1673 cm^{-1}) the C=O stretching at carbonyl groups at imino acids and glycine forming intramolecular hydrogen bonds with H atoms at both NH_2 and, unusual for proteins, CH_2 groups at glycine at a neighbouring chain and (iii) (~1640 cm^{-1}) the C=O stretching at carbonyl groups forming hydrogen bonds between two, often charged, amino acids as well as hydrogen bonds to water along the entire helix.

The IR spectrum of films prepared from diluted solutions ($c < 50$ μg ml^{-1}) corresponds to solution spectra indicating that native collagen molecules interact with water adsorbed on the titania surface. In films prepared from solutions ($c \geqslant 50$ μg ml^{-1}) collagen multilayers are formed. The amide I mode is blue-shifted by 18 cm^{-1}, indicating that intramolecular hydrogen bonds at imino acid rich fragments are weakened. Simultaneous red-shift of the amide A mode implies that the strength of hydrogen bonds at the imino acid poor fragments increases. Theoretically predicted distortion of the collagen structure upon adsorption on the titania surface is experimentally confirmed.

Introduction

Fibril forming collagens are the most common proteins in the animal kingdom [1,2]. They constitute more than 90% of the

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organic mass of bones and are the major component of connective tissues, tendons, skin, ligaments and cornea [2]. Due to their abundance in nature collagens find enormous industrial applications [3–5].

Since few decades, X-ray diffraction studies have been performed in order to solve the structure of fibril forming collagens [6–11]. Depending on the protein fragment used in X-ray studies, type of fibril forming collagen and sample preparation procedures, the structure of collagens varies between different reports [6,7,9,11–13]. It is well known that type I collagen is composed of three polypeptide chains coiled together to form a right handed helix [6,14]. Each polypeptide chain contains *ca.* 1000 amino acids which are built of three amino acids repeat unit: GXY. Glycine (Gly, G) is present in each repeat unit and constitutes exactly one third of all amino acids in the polypeptide chain [6,7,9,11]. It is essential to maintain the helical structure of the collagen molecule.

The X and Y positions are often occupied by two imino acids: proline (Pro, X) and hydroxyproline (Hyp,Y), respectively. They constitute *ca.* 20% of the total amino acid sequence of the collagen molecule. The high content of imino acids at the collagen molecule stabilizes the helix and contributes to a formation of hydrogen bonds unusual in other proteins [6–10]. In other words, the NH groups of Gly form intramolecular hydrogen bonds to carbonyl groups at Pro at the adjacent chain [6–10]. In a polypeptide chain, imine groups at Pro and Hyp do not contain a corresponding H atom and thus, they cannot participate in the formation of hydrogen bonds common in proteins. Interchain contacts between H atoms at CH groups of Gly and Hyp and the O atom at carbonyl groups at Gly and Pro at neighbouring chains were found [9]. The interatomic distances and angles between the C^{α}—H···O=C fragments, as demonstrated in [9], are suitable to describe these contacts as hydrogen bonds. Moreover, it was found that in the crystal state O atoms at carbonyl groups at Gly and Hyp can be also involved in water bridged hydrogen bonding either within a single polypeptide chain or between two chains [8,10].

Ionisable residues such as lysine (Lys), arginine (Arg), glutamic acid (Glu) or aspartic acid (Asp) constitute approximately 15–20% of all amino acids at the triple helix and around 40% of GXY triplets contain at least one charged residue at either X or Y position [7,11,15]. Charged amino acids are able to form hydrogen bonds either with an amino acid at the helix or with water molecules. Despite the fact that these hydrogen bonds are weak, they were found to stabilize the triple helix [11,15,16].

X-ray diffraction studies were performed either on fragments of the collagen molecule or collagen-like polymers [7,10–12,16]. Due to the analytical requirement of a crystal, the content of water in analyzed collagens is limited, affecting the resolved protein structure [16]. Therefore, the need of the use of other structure sensitive techniques aiming at the determination of the structure of the entire collagen molecule as a function of water content is required. Infrared spectroscopy (IRS), by contrast to X-ray diffraction, is not sensitive to the molecular structure at the atomic-level but it is sensitive to conformational changes in proteins upon functional transitions (*e.g.* protein aggregation, folding) or upon intermolecular interactions (*e.g.* binding, hydrogen bonding) [17–20]. The advantage of IRS is due to a wide spectral range covering vibrational frequencies from various functional groups present not only in a protein but also in its environment.

In the IR spectrum of proteins, the amide I mode has the most significant analytical relevance because, it contains the information about the secondary structure of a protein [18,19]. Collagen forms a coiled helix, thus according to the classical assignment of the amide I mode in proteins, one band centred between 1640 and 1630 cm^{-1} is expected in the IR spectrum [18,19]. However, the amide I mode of type I collagen in aqueous solutions has the maximum of absorption at 1655–1661 cm^{-1} [3,21–24]. This mode is asymmetric and composed of three to four superimposed absorption bands. Therefore, the amide I band of collagens have to be analyzed separately [21–23,25,26]. The assignment of the deconvoluted amide I modes of collagen to its structure is based on the spectral analysis of collagen-like polymers which correspond to imino acid rich protein fragments [21,26]. The deconvolution of the amide I mode of collagens and collagen-like polymers in aqueous solutions exhibits following absorption modes: 1693–83, 1665–1651 and ~1635 cm^{-1} [21–24]. In some IR spectra, a fourth absorption mode at 1644 cm^{-1} was observed [21,23]. The high frequency band is assigned to carbonyl groups not involved in hydrogen bonds [21]. The strongest amide I mode, located between 1650 and 1660 cm^{-1} is assigned to the C=O stretching mode at Pro hydrogen bonded to the NH group at Gly [21,23]. The low frequency modes (around 1635 cm^{-1} and 1644 cm^{-1}) are attributed to the C=O groups on the Gly and Hyp amino acids that are hydrogen bonded to water molecules [23,26]. Clearly, the assignment of the absorption modes contributing to the amide I mode does not represent the network of hydrogen bonds existing at the collagen triple helix [7–9,11,16]. Therefore, the analysis of the amide I mode requires a revision. In this paper, infrared spectroscopy and quantum chemical calculations at the density functional theory (DFT) level are used to analyze the amide I mode of collagen. The assignment of deconvoluted amide I modes described in this paper corresponds well to the molecular structure available from X-ray diffraction studies [7–9,11,12,16].

Fibril forming collagen molecules agglomerate to form large supramolecular assemblies. Moreover, the adsorption of proteins on implant surfaces is one of the most important steps in the biointegration process [27]. Therefore, the knowledge of the secondary structure of a protein and its changes due to the adsorption process is required to understand the process of protein folding and unfolding, the process of cell adhesion or processes occurring at implant surfaces. On the one hand IRS is sensitive to structural changes in proteins [18–20]. On the other hand reflection, based infrared techniques such as infrared reflection–absorption spectroscopy or attenuated total reflection spectroscopy are suitable to study molecular assemblies at solid surfaces [28–34]. Polarization modulation infrared reflection–absorption spectroscopy (PM IRRAS), due to the so called surface selection rule [35] is sensitive to submonolayer amounts of species adsorbed at a surface reflecting the IR radiation. The modulation of the polarization state of the incoming radiation cancels, in the spectrum, the contribution from the sample environment such as water vapor and CO_2 from the atmosphere [33,36]. The PM IRRAS is therefore an attractive structure analyzing method to study the adsorption process of collagen at solid surfaces. The spectral analysis of collagen adsorbed on the titania surface allows for a correlation of the protein structure with changes in the water content of the protein. For the first time in the literature, theoretically predicted structural changes in the collagen molecule due to adsorption on the titania surface [37,38] were confirmed.

Experimental

Substrate preparation

The substrate for the adsorption of collagen was a thin titanium layer deposited on a gold surface. First, a 7 nm thick adhesive layer of titanium was deposited on the glass surface. Next, a 200 nm thick gold film was deposited via plasma vacuum deposition on this adhesive layer. In the last step, the gold surface was covered by a 7 nm titanium layer. At the titanium surface ca. 4 nm thick titanium oxide layer is formed as described in [39]. The glass slides were cut into 25 × 20 mm^2 pieces.