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Impact of temperature and electrical potentials on the stability and structure of collagen adsorbed on the gold electrode

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ABSTRACT

The morphology and structure of collagen type I adsorbed on gold electrodes were studied as a function of electrode potential and temperature by means of capacitance measurements, polarization modulation infrared reflection–absorption spectroscopy and scanning force microscopy at temperatures of 37 °C, 43 °C and 50 °C. The selected temperatures corresponded to the normal body temperature, temperature of denaturation of collagen molecules and denaturation of collagen fibrils, respectively. Independently of the solution temperature, collagen was adsorbed on gold electrodes in the potential range $-0.7\ V < E < 0.4\ V$ vs. Ag/AgCl, where the protein film was very stable. Fragments of collagen molecules made a direct contact to the gold surface and water was present in the film. Protein molecules were oriented preferentially with their long axis towards the gold surface. Collagen molecules in the adsorbed state preserved their native triple helical structure even at temperatures corresponding to collagen denaturation in aqueous solutions. Application of $E < -0.75$ V vs. Ag/AgCl leads to the swelling of the protein film by water and desorption from the electrode surface. IR spectra provided no evidence of the thermal denaturation of adsorbed collagen molecules. A temperature increase to 50 °C leads to a distortion of the collagen film. The processes of aggregation and fibrilization were preferred over thermal denaturation for collagen adsorbed on the electrode surface and exposed to changing potentials.

1. Introduction

Collagens belong to the most abundant proteins in animals. They are the major protein component of the extracellular matrix, constitute the organic mass of bones, and are the major component of connective tissues, tendons, skin, ligaments and cornea [1,2]. Twenty-one types of collagen have been identified and described in the literature [2]. Fibril forming collagens include types I, II, III, V and XI. Among them, type I collagen is the most abundant [2]. In type I collagen, three polypeptide chains are coiled together to form a right-handed helix [3,4]. Each polypeptide chain contains ca. 1000 amino acids built of a three amino acid sequence GXY. Glycine (Gly, G) appears at every third position and is essential to maintain the helical structure of the collagen molecule [3,5–7]. X and Y positions of the polypeptide chain are often occupied by two imino acid proline (Pro, X) and hydroxyproline (Hyp, Y). They constitute around 20% of the total amino acid sequence of the collagen molecule. Imino acid-rich fragments of the collagen molecule stabilize the helix, form a unique set of hydrogen bonds and determine its structure [3,5,6,8,9]. According to X-ray diffraction studies [3,5,6,8,9], the NH group at Gly forms hydrogen bonds only to the carbonyl groups of Pro. At the collagen helix, the lack of NH groups at Pro and Hyp disables the formation of hydrogen bonds to O atoms at C═O groups of Gly and Hyp. The high content of imino acids in collagen leads to the formation of unique sets of hydrogen bonds not found in other proteins. The O atom of the carbonyl group of Gly or Pro at one polypeptide chain makes contacts to H atoms at the methylene group of Gly at the adjacent chain [6]. The interatomic distances and angles between the $C^{\alpha}-H\cdots O{=}C$ fragments justify a description of these contacts as hydrogen bonds. Carbonyl groups at Gly and Hyp are involved in the formation of water-bridged hydrogen bonds [8,9]. Ionisable residues such as lysine (Lys), arginine (Arg), glutamic acid (Glu) or aspartic acid (Asp) are found at imino acid-poor fragments of the collagen helix [5,7,10]. They constitute approximately 15–20% of all amino acids in the triple helix and around 40% of GXY triplets contain at least one charged residue. Charged amino acids form hydrogen bonds between themselves or with water molecules [11]. Despite the fact that these hydrogen bonds are labile, they stabilize the triple helix [7,10,11].

The network of hydrogen bonds at the collagen helix as well as the hydration shell of the collagen molecule depends on the temperature, water access, aggregation and progress of the fibrilization process [10,12–16]. Temperature changes of the electrolyte solution containing collagen monomers lead to two competing processes: (i) thermal denaturation and dissolution of the triple helix of collagen into single disordered polypeptide chains or (ii) aggregation of collagen molecules and formation of collagen fibrils [12,14–18]. Calorimetric studies show that in aqueous solutions at the temperature range 37–42 °C thermal

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denaturation of the triple helix collagen monomers takes place [16]. It is initiated at the so called labile domains. At these fragments new hydrogen bonds between amino acids and water are formed destabilizing the triple helix finally leading to its distortion and separation into three disordered chains. Thermal denaturation of collagen monomers leads to the formation of gelatine. Depending on the pH of the electrolyte solution and the ionic strength of the electrolyte, collagen molecules may aggregate spontaneously to form fibrils. This process is most efficient in the temperature range 26–37 °C before thermal denaturation of collagen commences [19–21]. Recently, it was demonstrated that collagen molecules adsorbed on solid surfaces may aggregate to fibrils at temperatures as low as 4 °C [18]. The progress of the fibrilization process depends not only on the temperature but also on the hydrophilicity of the used substrate. Due to the dense packing of collagen molecules, the amount of water is low in fibrils. Therefore, the set of hydrogen bonds may differ between collagen fibrils and collagen monomers. Collagen fibrils are thermally more stable than monomers. They undergo denaturation at 50 °C [16].

X-ray diffraction belongs to the most important analytical techniques used for the determination of the structure of proteins [22]. The biggest limitation of this technique arises from the need to investigate a crystal rather than the structure in solution that may differ substantially from the crystal structure. Moreover, dynamic changes at proteins such as thermal denaturation, self-aggregation, and interaction with other proteins or cell membranes cannot be studied by means of diffraction techniques. For these purposes, infrared spectroscopy (IRS) is widely used. It is sensitive to conformational changes in proteins upon functional transitions (e.g. thermal denaturation, protein aggregation, folding) and upon intermolecular interactions (e.g. binding, hydrogen bonding), but lacks atomic-scale sensitivity to the protein structure [23–26]. The IR spectrum of collagen differs from the IR spectra of most globular and transmembrane proteins due to its unique amino acid sequence, the high content of imino acids and the unique network of hydrogen bonds [12,13,15,27,28]. The amide I mode of proteins is of large analytical importance because; it provides the information about the secondary structure of a protein [24,25]. This spectral region is particularly interesting for collagen films since the collagen triple helix yields quite different amide I bands than other proteins [12,13,24,25,27]. Collagen molecules form a coiled helix, thus based on the classical analysis of the amide I mode of proteins, a single mode between 1640 and 1630 cm^{-1} should appear in the IR spectrum [24,25]. Interestingly, the amide I mode of the type I collagen is centered at 1655 cm^{-1} and is composed of three or four superimposed absorption bands [12,13,27,28]. The deconvolution of the amide I band of collagen gives the following components: a weak mode centered at 1693–1683 cm^{-1}, the strongest band at 1660–1651 cm^{-1} and one or two modes between 1644 and 1630 cm^{-1} [12,13,27,28]. The amide I mode reflects the complex network of hydrogen bonds existing at the collagen helix. Therefore, one may expect large changes in the amide I mode region of collagen when the hydrogen bonding network is disturbed by external impulses such as temperature, electric potentials, coordination of electrolyte ions or dehydration. According to the literature, temperature-dependent changes in the amide I mode are marginal, complicating the analysis of the IR spectra of collagens [12,13,15]. Upon thermal denaturation, the positions of the deconvoluted amide I modes do not shift [13,29]. The ratio of the integrated intensity of the amide I mode at 1655 to 1635 cm^{-1} changes from >1 to <1 in the native and denaturated collagen, respectively [13]. This change is attributed to the cleavage of hydrogen bonds at imino acid-rich fragments of collagen and formation of new hydrogen bonds to water. Fibril formation leads to a small change in the shape of the amide I mode of collagen in comparison to the amide I mode of monomers [12,15]. The intensity of the entire amide I mode decreases, it becomes broader and a shoulder at the low frequency side becomes apparent. The main amide I mode undergoes a small red-shift from 1655 to 1651 cm^{-1} [15]. The described changes indicate that collagen maintains the triple-helical structure during fibrilization, in which only small changes of internal hydrogen bonds of the collagen molecule are involved.

A change of temperature, ionic strength or pH of the electrolyte may lead either to the denaturation or fibrilization of collagen molecules. These two processes may compete with each another. The outcome may affect post-implantation processes of tissue development and regeneration after medical surgery or may cause metabolic abnormalities leading to pathogenic conditions [30]. It is difficult to differentiate between these processes by experimental techniques. Since the process of collagen aggregation depends not only on the temperature but also on the ionic strength and pH of the electrolyte solution [19–21], electric fields appearing at the interface should play an important role in the process of collagen self-assembly or denaturation. The effects of electric fields acting at the electrode (implant surface) on the adsorbed collagen have not yet been described in the literature. In this contribution electrochemistry, IR spectroscopy and SFM under electrochemical control are used to study the simultaneous impact of temperature and changing electric fields on type I collagen adsorption, desorption, denaturation and aggregation on a model metal surface.

2. Materials and methods

2.1. Substrate preparation

Polycrystalline gold disks (diameters 4 and 15 mm) as well as 100 nm thick gold films evaporated on a glass were used as substrates for collagen adsorption. Before the self-assembly of the protein, gold disks were washed in water and flame annealed. Gold slides were washed with water and ethanol and cleaned in ozone. Collagen I from a rat tail dissolved in 0.02 mol l^{-1} acetic acid solution was purchased from BD Biosciences,Heidelberg, Germany. The diluted collagen solution containing 100 μg ml^{-1} of the protein was prepared in home-made phosphate buffered saline solution, pH = 7.4 (PBS). The process of the self-assembly of collagen on the Au surface was carried at a constant temperature $T = 4$ °C. At this temperature collagen molecules were adsorbed on the Au surface without formation of any undefined structure of the protein aggregates. The time of self-assembly was 10 min. The modified Au surface was rinsed with deionized water and dried under flow of argon. According to ellipsometric measurements the collagen film on the Au surface has a thickness of 6.6 ± 0.3 nm [31]. Collagen films prepared according to this procedure were stable for several weeks.

2.2. Electrochemical measurements

A CHI660A potentiostat (CH Instruments, Austin, USA) with the corresponding software was used to perform the electrochemical measurements. A three electrode cell was assembled with polycrystalline Au disc electrode as the working electrode, a platinum sheet as auxiliary electrode and a Ag|AgCl|3 M KCl as reference electrode (denoted here for brevity as Ag|AgCl) in an all-glass thermostated vessel. The reference electrode was separated from the electrolyte solution via a salt bridge. The electrolyte was 0.1 M NaF (Merck, Darmstadt, Germany). The measurements were done for collagen films self-assembled on the Au electrode at $T = 4$ °C. Prior to the experiment, the cell was purged with argon for 30 min and the temperature of the electrolyte was set to constant values of 37 °C, 43 °C or 50 °C. The cleanness of the electrochemical cell was tested by recording cyclic voltammograms in the electrolyte solution. The AC voltammograms were recorded in positive and negative directions with a scan rate of 5 mV s^{-1} and an AC perturbation with 10 mV amplitude and 20 Hz frequency. The capacitance vs. potential curves were calculated from the in-phase and out-of-phase components of the current assuming that the electrochemical cell behaved equivalent to a resistor in series with a capacitor.