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Adsorption of amino acids on hydrophilic surfaces

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Abstract

Sum frequency generation vibrational spectroscopy (SFG) is a powerful tool for *in situ* investigation of adsorption processes at biologically important solid–liquid interfaces. In this work adsorption of selected amino acids on fused silica, calcium fluoride and titanium dioxide substrates was studied by this technique. SFG spectra taken at the amino acid solution–fused SiO_2 interface revealed the lack of formation of any ordered adsorbate layer, regardless of whether acidic or other, e.g. aromatic, amino acids were used. *Ex situ* spectra (measured after drying the substrate) showed the formation and gradual growth of amino acid crystallites. In the case of CaF_2, growth of randomly oriented aspartic acid crystallites was observed even at the solution–substrate interface. Finally, on the TiO_2 substrate, acidic amino acids formed a stable, uniform, more or less ordered coating, which remained unchanged even after drying the sample. On the other hand, non-acidic amino acids like phenylalanine showed very little affinity towards TiO_2, emphasizing the role of the acidic side chain in the bonding to the substrate. The fact that formation of an amino acid overlayer was observed only on titanium dioxide is probably related to its biocompatibility property.

1. Introduction

It is general knowledge that when an implant is inserted into a living organism, adsorption of water and biomolecules including peptides and plasma proteins is the first event of the interaction of the biological system with the foreign material. The properties of this initially formed biofilm largely determine bonding and growth of body cells on the foreign surface, and thus the fate of the implant in the biological environment. Due to its importance not only in biomaterials research but also in biofouling control or food processing, structural properties of the biofilm are extensively studied [1, 2].

Amino acids, the building blocks of peptides and proteins, belong to the simplest biomolecules. Investigation of their behavior on biomaterial surfaces is an essential step in understanding biomolecular adsorption phenomena. Apart from their role in protein–surface interactions [2, 3], amino acids offer exciting possibilities for functionalization of solid surfaces due to their multifunctionality and tendency for self-organization [4].

The bonding and the structure of amino acid monolayers deposited onto clean and ordered metal or metal oxide surfaces can be successfully investigated with modern surface science tools including electron and optical spectroscopic methods in ultrahigh vacuum environments [4]. The available results indicate that amino acid molecules, which are zwitterionic in the crystalline phase, adsorb dissociatively on metal single-crystal surfaces. Bonding is usually accomplished through the deprotonated carboxyl group, coordinated either monodentally or bidentally to surface metal atoms. The amino group, which is protonated in the zwitterionic form, typically becomes neutral and is often involved in bonding [4]. On metal oxides like the (2×1) reconstructed $TiO_2(110)$ surface, glycine (Gly), the simplest amino acid, tends to decompose by losing its amino functionality when adsorbed in submonolayer quantities [5]. This conclusion is partly supported by desorption experiments [6], although clear signs of glycinate monolayer formation were observed on $TiO_2(110)$ (1×1) by scanning tunneling microscopy [7]. On a defect-free $TiO_2(001)$ surface, proline was observed to bond through its deprotonated carboxylate group, while on a heavily ion-bombarded surface significant decomposition was found [8].

The situation seems to be very different in wet environments. In general, the interaction between the amino acids and the metal or metal oxide surface is significantly

weaker in a solution than under UHV conditions. For example, on TiO_2 no glycine or glutamine adsorption was found from solution [9, 10]. These data as well as simulation results [11] suggest that most amino acid adsorbates are bonded through much weaker interactions like electrostatic attraction or hydrogen bonding than in a UHV environment. A notable exception is the case of the acidic amino acids aspartic acid (Asp) and glutamic acid (Glu), which were found to form somewhat stronger bonds with oxidized Ti [10, 12] or stainless steel [13] surfaces. The nature of these bonds, however, is still controversial: adsorption/desorption kinetics data were interpreted on the basis of bonding through the amino functionality [12] while spectroscopic data suggest that the carboxylic groups are also involved [10, 13].

A general difficulty in investigation of biomolecule adsorption in wet environments is that the arsenal of surface specific analytical tools used in UHV experiments is not applicable. Although the adsorbed amount of the biomolecule can be relatively accurately measured [12, 13], spectroscopic information is much more complicated to obtain. Infrared and Raman spectroscopy, for example, are not surface specific; therefore subtraction of signals due to dissolved biomolecules or the solvent itself usually requires special efforts. A possibility for overcoming this limitation is to apply nonlinear optical spectroscopy techniques. For example, sum frequency generation, a second-order nonlinear optical process, is forbidden in the bulk of centrosymmetric media, but becomes allowed at interfaces, where centrosymmetry is necessarily broken. A spectroscopic application of this process is sum frequency generation vibrational spectroscopy (SFG). In a SFG experiment the sample is irradiated with a fixed frequency visible and a tunable infrared light beam, and a coherently generated beam with a frequency corresponding to the sum of the frequencies of the exciting beams is detected. By tuning the frequency of the infrared excitation, the vibrational spectrum of the species in the interfacial region can be measured. The amplitude of the SFG signal at vibrational resonances is connected to the surface density and ordering of the interfacial functional groups. Due to its inherent surface specificity and submonolayer sensitivity, SFG turned out to be an excellent tool for studying interfacial phenomena at gas–solid, liquid–solid and even liquid–liquid interfaces [14–18]. Further information about the theory and practice of SFG spectroscopy can be found in the works cited above.

In this study SFG is used to explore the adsorption of selected amino acids at the solid–amino acid interface. The primary focus is on the behavior of acidic amino acids, due to their relatively strong affinity towards solids in wet environments, as outlined above. The substrates studied included fused silica, calcium fluoride, which is an important component of tooth enamel as well as dental ceramics, and titanium dioxide, a well-known representative biocompatible material. Since the level of hydroxylation of the substrate seems to be important from the point of view of amino acid adsorption [11], all samples were specially pretreated to ensure a highly hydrophilic initial surface.

2. Experimental details

L-aspartic acid was purchased from Fluka (Germany); all other amino acids were obtained from Reanal (Hungary). The amino acids were dissolved in D_2O from Cambridge Isotope Laboratories (USA) to avoid complications due to strong interfacial water signals. Adsorption experiments were done in the 0.1–5 mg ml^{-1} concentration range. The pH of the solution was controlled by adding NaOH or HCl as required. The ionic strength was kept at 20 mM during the concentration dependent measurements by adding NaCl to the solution.

Sum frequency generation measurements were carried out in a near total internal reflection geometry as described in [19], where the angle of incidence of the relevant light beams at the interface studied is very close to the critical angle for total reflection. Accordingly, the substrate on which the adsorption experiments were carried out was prepared on the face bordered by the leg of a right angle prism. IR-grade fused silica and CaF_2 prisms (single crystal with unspecified orientation) were purchased from Crystaltechno Ltd (Russia), and the carefully cleaned faces of the prisms were used as substrates. The TiO_2 substrate was prepared by evaporation of a 40–50 nm TiO_2 layer on the appropriate face of a fused silica prism. The resulting film consists of stoichiometric TiO_2, as confirmed by x-ray photoelectron spectroscopy (XPS) measurements, in the form of anatase crystals and amorphous titanium dioxide.

Substrates exposed to ambient air are covered by a 2–3 nm thick layer consisting of adsorbed hydrocarbons. Since this contamination layer interferes with adsorption experiments, a cleaning procedure needs to be applied which also ensures reproducible initial surface conditions. In this work surface cleaning was achieved by washing of the samples in chloroform and exposing them in air to the ozone generating ultraviolet light of a mercury discharge lamp in a commercial UV–ozone apparatus (Tipcleaner™ by Bioforce Nanosciences, USA). This treatment leads to total oxidation of the hydrocarbon contamination and results in a hydrocarbon-free, very hydrophilic surface [20] indicating the high density of surface hydroxyl groups on all three substrates.

Adsorption of amino acids was followed by SFG at the substrate–amino acid solution interface. For these measurements the cleaned prisms were placed (with the face to be used as substrate down) onto Teflon cells filled with the amino acid solution. SFG spectra presented here were recorded in the region of C–H and O–H stretches (2700–3800 cm^{-1}). The spectra were usually measured in the ppp polarization combination (both exciting as well as the SFG signal light beams p-polarized) unless otherwise indicated. The detailed description of our SFG spectrometer was given in [21] and will not be repeated here. In certain cases SFG measurements were carried out at the air–substrate interface, after drying the surface exposed to amino acids with a stream of dry N_2. For these investigations the near total reflection geometry required one to use the prism face next to the hypotenuse as substrate. It is worth noting that since both exciting photon energies lie well below the band gap of TiO_2, no photocatalytic effects are expected in our experiments.

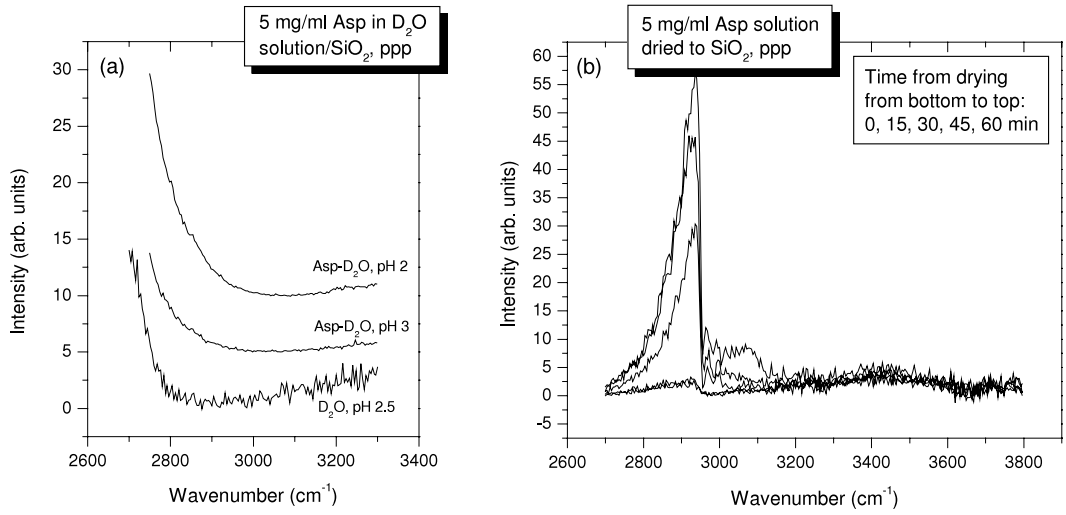

Figure 1. (a) SFG spectra of the heavy water–fused silica interface at pH 2.5, and 5 mg ml^{-1} Asp solution–fused silica interface at pH 2 and 3. Spectra were shifted vertically for clarity. (b) Time dependent changes of the SFG spectrum of the fused silica surface after removal from the 5 mg ml^{-1} Asp solution at a 'strong' location. The time of contact with the solution was 60 min. All spectra were taken in the ppp polarization combination.

Control experiments concerning the cleanliness and composition of the substrates and the adsorbed amounts of amino acids were carried out by means of XPS using our surface analysis system made by Omicron Nanotechnology (Germany). The photoelectrons were excited by Al Kα photons (1486.7 eV) and were analyzed in the constant analyzer energy mode at 30 eV pass energy.

3. Results and discussion

3.1. Interaction of amino acids with fused silica

In figure 1 SFG spectra of aspartic acid on fused silica are presented at the solution–SiO_2 interface (a) and, after drying the sample, at the air–SiO_2 interface (b) in the range of the C–H (and O–H) vibrations. In the spectrum of the D_2O–fused silica interface the increasing signal towards smaller wavenumbers is due to the high wavenumber tail of the O–D stretches of the heavy water molecules oriented by the substrate. Figure 1(a) shows clearly that addition of aspartic acid to the solution has hardly any effect on the spectrum. This result indicates that adsorption of aspartic acid to fused silica is rather weak.

In contrast, if the sample is removed from the amino acid solution and dried in a nitrogen stream, interesting time dependent evolution of the SFG spectra was observed (figure 1(b)). Immediately after drying, the surface was quite homogeneous with very small hydrocarbon signals (spectra at 0–15 min). After some time, at certain locations, unusually strong hydrocarbon signals appeared (spectrum at 30 min), which continuously increased (45 and 60 min). At other parts of the sample much smaller intensities were detected.

To elucidate the origin of these signals, spectra were taken with various polarization combinations. It was found that not only the ppp and ssp but very often the spp and psp spectra also exhibited comparable signal intensities. It is well established that an SFG signal is expected only in the ssp, ppp, sps and pss polarization combinations for azimuthally isotropic layers [22]. In the case of chiral adsorbates, signals in the spp, psp and pps polarization combination can also be detected, but usually at much weaker intensity than in the above four polarization combinations [23].

Control experiments show that the contamination layer developing on the cleaned and pure water exposed SiO_2 is homogeneous, its SFG intensity is always much weaker than that in the 'strong' spots of the aspartic acid treated samples and the signal can only be obtained in the 'normal' polarization combinations.

Taking into account that the dried surface was highly inhomogeneous, at the spots with strong SFG signals the intensities of the 'normal' and 'chiral' polarization combinations were comparable (but varying from location to location) and the signal intensity exhibited a characteristic time dependence, we can conclude that the signals can be attributed to growing aspartic acid crystallites. Indeed, aspartic acid crystallizes in the non-centrosymmetric monoclinic C_2–$P2_1$ space group [24], in which bulk sum frequency generation is allowed. In addition, the C–H vibration frequencies are in good agreement with the peak positions expected for aspartic acid (see below).

This observation also confirms that aspartic acid interacts weakly with SiO_2. The aspartic acid molecules which are in the vicinity of the fused silica surface when drying is accomplished remain mobile on the surface and can readily condense into randomly oriented crystallites. Very similar results were obtained if other amino acids including glutamic acid, glutamine, cysteine or phenylalanine were studied. On the basis of our experimental results, we can conclude that the interaction between amino acids and fused silica is in general very weak.

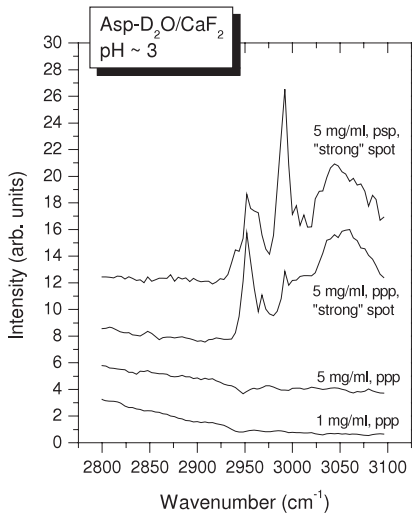

Figure 2. SFG spectra measured at the CaF_2–aspartic acid solution (in D_2O) interface at different concentrations and locations. Spectra were displaced vertically for clarity.

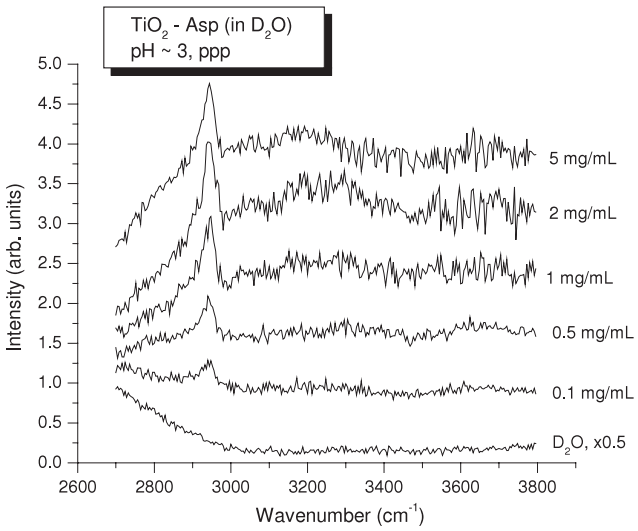

Figure 3. SFG spectra collected at the Asp solution (in the D_2O)–TiO_2 interface at different aspartic acid concentrations. The spectra were displaced vertically for better visibility.

3.2. Adsorption of aspartic acid at the CaF_2–solution interface

Somewhat different behavior can be observed on the UV–ozone treated CaF_2 substrate. Figure 2 shows SFG spectra measured at the CaF_2–aspartic acid solution interface at different solute concentrations and locations. At 1 mg ml^{-1} concentration the interface is quite homogeneous with a weak negative peak around 2940 cm^{-1} indicating the presence of adsorbed aspartic acid molecules. At larger amino acid concentrations, however, a strongly inhomogeneous interface was obtained.

For example, at certain locations the spectrum of the 5 mg ml^{-1} solution–calcium fluoride interface resembles that of the more dilute solution, while at other spots rather intense signals were measured. At these 'strong' locations intense signals were obtained also in the 'chiral' polarization combinations, indicating the formation of aspartic acid crystallites at the solution–substrate interface. At an intermediate concentration of 2 mg ml^{-1} similar behavior was seen, although the intensity from the aspartic acid crystallites was weaker.

The results show that the interaction between aspartic acid and CaF_2 is somewhat stronger than in the case of SiO_2. At low concentrations there are clear signs of the presence of adsorbed molecules at the interface. Detailed analysis of the adsorbates is, however, difficult, since UV–ozone treated CaF_2 provides nucleation sites for formation of aspartic acid crystallites, whose strong bulk SFG signals completely dominate the spectrum.

3.3. Amino acid adsorbates on TiO_2

Aspartic acid exhibited markedly different adsorption behavior on TiO_2. SFG spectra collected at the TiO_2–solution interface are shown in figure 3 as a function of the solute concentration.

This interface turned out to be homogeneous, without 'intense' or 'weak' spots. The rising signal level at lower wavenumbers in the spectrum of the TiO_2–D_2O interface (bottom trace) is again due to the tail of the O–D vibrations. At the lowest aspartic acid concentration studied this tail almost completely disappeared, indicating that the ordering of the heavy water molecules became disrupted, and a peak appeared at 2944 cm^{-1}. Upon increasing the concentration of the aspartic acid, the intensity of this C–H originated peak slowly increased, along with the increase of the nonresonant baseline which exhibited a cut-off at low wavenumbers. No SFG signal was obtained in any other polarization combination.

Aspartic acid has two functional groups which exhibit C–H stretching vibrations: the methyne hydrogen attached to the α carbon atom and the methylene group adjacent to the α carbon atom. Indeed, in the SFG spectra of Asp crystallites grown at the SiO_2–air and especially the CaF_2–solution interface, three distinct narrow peaks can be seen at 2940–2950, 2965–2970 and 2990–2995 cm^{-1}. Assignment of the methyne stretch seems somewhat controversial, as it was attributed to a vibration either in the range 2970–2980 cm^{-1} (zwitterionic asparagine [25]) or in the range 2990–3000 cm^{-1} (zwitterionic serine in water [26] or aspartic acid in solid [27]). If one accepts the latter assignment then the 2940 cm^{-1} peak can be attributed to the symmetric and the 2965 cm^{-1} one to the antisymmetric methylene vibration, in qualitative agreement with the literature data [27, 28].

Accordingly, the C–H peak in the SFG spectra of the Asp solution–TiO_2 interface can be assigned to the symmetric methylene stretch.

It is worth noting that although the solute concentration was changed by a factor of 50, the methylene signal increased only by 2–3 times. This clearly indicates that the observed signal is due to molecules adsorbed at the interface, as a signal from the bulk of the solution is expected to be much more sensitive to the bulk concentration (SFG intensity is proportional to the square of the number density if orientation effects are neglected).

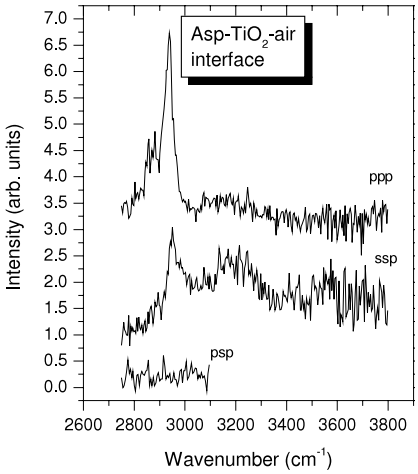

Figure 4. SFG spectrum of the TiO_2–air interface after Asp adsorption from a 1 mg ml^{-1} solution for 45 min at pH 3. Spectra were shifted vertically for clarity.

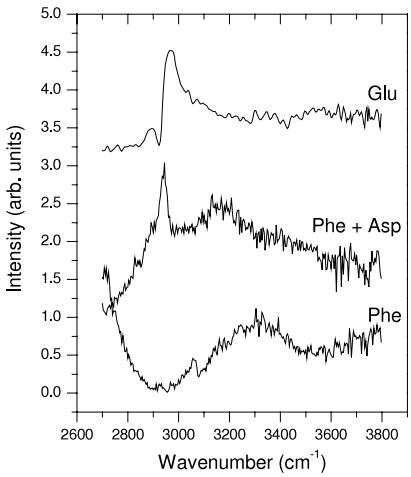

Figure 5. SFG spectrum of the 1 mg ml^{-1} Phe–D_2O solution–TiO_2 interface at pH 5 (bottom curve) and the 1 mg ml^{-1} Asp + 1 mg ml^{-1} Phe–D_2O solution–TiO_2 interface at pH 3 (middle curve). The spectrum of the 1 mg ml^{-1} Glu–D_2O solution–TiO_2 interface at pH 3 is also shown (the top curve). Spectra were shifted vertically for clarity. All spectra were taken in the ppp polarization combination.

Another important observation is that the adsorbed Asp layer forms very rapidly. The time between contacting the substrate with the amino acid solution and taking the first spectrum is not more than 1–2 min. According to our experience, the Asp layer fully develops during this time with no noticeable change even after hours.

By using the basic equations of SFG (e.g. [22]), it is easy to see that in the total internal reflection geometry we measure only the *zzz* component of the surface susceptibility tensor in the ppp polarization combination. Taking into account the hyperpolarizability properties of a methylene group (see e.g. [29]), we can conclude that the strong dominance of the symmetric methylene signal in our spectrum is qualitatively compatible with an orientation distribution in which the symmetry axes of the methylene groups are more or less parallel with the surface normal. This orientation can be realized by coordinating both carboxylic groups of the Asp molecule to the TiO_2 surface, as proposed in [10, 13].

In clear contrast to the case for experiments on the other two substrates, no signs of crystallite formation were observed even after drying the Asp adsorbates on TiO_2. The substrate–air interface remained homogeneous, with the dominating contribution to its SFG spectrum still from the symmetric methylene stretch. No signal was detected in the 'chiral' polarization combinations. As an example, in figure 4 the spectrum of a TiO_2 substrate exposed to a 1 mg ml^{-1} solution is presented after drying. The small peaks around 2850 and 2880 cm^{-1} originate from the hydrocarbon contamination collected by the sample after finishing the adsorption experiment. XPS control measurements revealed that the amount of Asp bonded to the substrate is in the order of one monolayer.

Turning to other amino acids, L-glutamic acid exhibits very similar behavior to aspartic acid when in contact with TiO_2: SFG measurements reveal a homogeneous, relatively ordered interfacial adsorbate structure both at the solution–solid (figure 5, upper spectrum) and at the adsorbate covered solid–air interface. Because of the higher number of C–H modes, the spectrum is more complicated than in the case of Asp. The observed behavior is in good agreement with previous studies, where Glu was found to be the amino acid most readily adsorbing on TiO_2 [10].

The SFG spectrum of the L-phenylalanine solution–TiO_2 interface taken at a relatively high amino acid concentration suggests that Phe adsorption is rather weak (figure 5, lower trace). The spectrum closely resembles that of the D_2O–TiO_2 interface (the broad structure around 3300 cm^{-1} is probably due to H_2O contamination), apart from a tiny aromatic signal at 3060 cm^{-1}.

If adsorption takes place from the mixture of Asp and Phe dissolved in D_2O, only Asp vibrations are detected in the interfacial spectrum, indicating that only the molecule with the acidic side chain has significant affinity towards TiO_2. The results presented suggest that coordination of the side chain carboxylic group to the surface is the crucial point in the formation of an ordered adsorbate layer at the TiO_2–amino acid solution interface.

4. Conclusion

In this work, interaction of selected amino acids was investigated with hydrophilized fused silica, calcium fluoride and titanium dioxide substrates by sum frequency generation vibrational spectroscopy. It was demonstrated that amino acid adsorption can be observed *in situ*, at the solution–solid interface in the near total internal reflection geometry.

The interaction between the amino acids and substrates studied was generally weak. No sign of amino acid adsorption was observed at the solution–fused silica interface, while growth of amino acid crystallites was seen after drying the substrate. The aspartic acid–calcium fluoride interaction was characterized by nucleation and growth of Asp crystallites even at the solution–solid interface. The only exception where

stronger adsorption processes were found was the case of titanium dioxide interacting with acidic amino acids. In the latter systems, formation of homogeneous, ordered adsorbate monolayers was observed in a wide concentration range, while non-acidic amino acids only weakly adsorbed on TiO_2. The presence of the acidic side chain is, therefore, of central importance in the formation of an adsorbed amino acid layer. The ability of TiO_2 to maintain this layer is probably related to its biocompatibility property.

Acknowledgments

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