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Surgar coating can stabilize the “native” structure and control the orientation of surface-immobilized peptides in air, providing a potential approach to retain biological functions of surface-immobilized biomolecules in air. This method is general and applicable to complex enzymes.

Water is typically required to maintain both the structure and activity of biological molecules such as proteins and peptides. Removal of water often destroys the native conformation of a protein or peptide, especially at ambient (room temperature or RT) or high temperatures. Therefore, maintaining the structure and function of proteins and peptides under harsh conditions, e.g., no or minimal water, is very challenging, but is very important for some applications such as biosensors and biofuel cells. Previous studies have shown that many solvent additives, e.g. sugars and poly alcohols, can retain the function of biomolecules and reduce the extent of denaturation during or after drying processes. Sugar stabilization of proteins in solution has been well established for more than twenty years. However, most studies describing sugar stabilization of proteins have been performed in bulk conditions and they largely focused on the variation of the protein storage conditions modified with sugar additives. Few studies have investigated surface-immobilized proteins and protein/sugar interactions at the molecular level in air.

An in-depth understanding of the structure, e.g., conformation and orientation of molecules at interfaces is very important in many fields, including antimicrobial coatings, electronic materials, sensors and bioensors, catalysis, and energy storage/production. The conformation and orientation of biological molecules at interfaces have been controlled and maintained by various methods. However, such methods have not been able to maintain the structure and orientation of proteins in a dry condition. We hypothesized that with the help of sugar molecules, we may be able to retain the conformation and orientation of surface immobilized biomolecules in air as they could do for biomolecules in the bulk environment.

Since the structure of a protein is complex, probing the effects of sugars on protein structure in detail can be challenging. Moreover, during protein drying processes, low temperatures, such as -45 °C, are commonly required. A method that is capable of operating under RT and maintaining the structure/function of peptide/protein during/after a drying process would be more broadly applicable. Herein, we present a simple method to control the orientation of a peptide immobilized on a surface by simply spin coating sugars on the immobilized peptide at RT. We have investigated the molecular structure of such a peptide in dry conditions at RT using Sum Frequency Generation vibrational spectroscopy (SFG), with the help of circular dichroic spectroscopy (CD). SFG has been widely used to study molecular structures of surfaces and interfaces, including peptides and proteins. Here we use peptides with well-defined secondary structures as models to investigate the interactions between sugar molecules and proteins in dry conditions. The method developed is general and can be applied to more complicated molecules such as proteins including enzymes, which are widely used for many applications such as biosensors, biochips, and biofuel cells.

A previous SFG study demonstrated that the N-terminus azido mutated MSI-78 (nMSI-78) peptide adopts (when in contact with aqueous phosphate buffer (PB)) an alpha helical conformation and is oriented with a small tilt angle relative to the surface normal after immobilization on an alkyne-terminated SAM surface through a click reaction. This nMSI-78 peptide is used as a model in the current study. As shown in Figure 1a, a peak centered near 1650 cm⁻¹ was detected in the SFG ppp (p-polarized SFG, p-polarized visible beam, and p-polarized IR beam) spectrum collected from the immobilized peptide/PB interface, which is assigned to the amide I vibrational mode of the alpha-helical structure. This peak disappears when the immobilized peptide was removed from the PB (Figure 1b and 1c). The disappearance of the peak indicates that the alpha helical structure of the peptide either transformed into a random coiled structure, or that the alpha-helical peptide lay down with a large tilt angle at the surface. CD spectra collected from the immobilized peptides exposed to buffer and air are similar though not identical (Figure S2). However, both CD spectra show helical structures. Therefore, we interpret the absence of SFG amide I signal from the surface immobilized nMSI-78 in air to be due mainly to an orientation of the alpha helical peptide that is parallel to the surface.

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To retain the SFG signals detected from the immobilized peptides, a layer of sugar was applied to the immobilized peptides in air. Sucrose, which has been widely used to stabilize proteins, was chosen as the sugar coating. When a spin-coated sucrose layer of sugar was applied to the immobilized peptides in air, the SFG signals detected from the immobilized peptides, a layer of sugar was applied to the immobilized peptides in air. Sucrose, which has been widely used to stabilize proteins, was chosen as the sugar coating. When a spin-coated sucrose layer using 200 mM sucrose solution was deposited over the peptide, the secondary structure of the surface immobilized nMSI-78 was similar to that measured in PB, as demonstrated by the detection of an SFG signal centered at 1650 cm\(^{-1}\) in the SFG ppp spectrum (Figure 1d). The SFG intensity decrease observed in Figure 1d (compared to that in Figure 1a) is due to the refractive index difference between PB and sugar (Table S1), and/or the orientation change of the immobilized peptides. The time-dependent SFG signal detected at 1650 cm\(^{-1}\) exhibited no noticeable change within 40 min of surface preparation (Figure 1d) which indicates that the spin-coated sucrose layer is stable at RT and that the molecular structure of the peptides was retained in the dry condition due to the interactions with the overlying protective sugar layer. CD spectroscopic data further showed that the immobilized nMSI-78 with a sugar coating on top adopts an alpha-helical structure (Figure S2).

We further investigated the effect on the peptide orientation in air of the sugar concentration in the spin-coated solution. Sucrose solutions with concentrations ranging from 25 to 325 mM were spin coated on a surface presenting the immobilized peptide, and then SFG ssp and ppp spectra were collected from the immobilized peptides (Figure S3). An amide I peak centered at 1650 cm\(^{-1}\) was observed in each SFG spectrum, indicating that the immobilized peptide adopted an alpha helical structure with sucrose coating, regardless of the sucrose solution concentration. The SFG spectra collected using different polarization combinations (ssp and ppp) were then used to deduce the orientation of the peptide. SFG orientational analysis of the alpha helical structure has been studied in detail before.\(^{[42-43]}\) The orientation of an alpha helical peptide on a surface can be defined by a tilt angle \(\theta\), the angle between the axis of peptide relative to the surface normal. The relationship between the measured \(x_{ppp}/x_{ssp}\) ratio and the tilt angle \(\theta\) can be plotted using the previously reported method by assuming the orientation angle \(\theta\) adopts a \(\delta\)-distribution (meaning that all peptides adopt an identical orientation). Detailed descriptions of the orientation analysis can be found in previous publications and will not be repeated here.\(^{[22-43]}\) Experimentally measured \(x_{ppp}/x_{ssp}\) ratios of the sugar-coated, immobilized peptides in air (prepared using different sugar solution concentrations) are summarized in Table S2 and are plotted in Figure 2a. For comparison purposes, the measured \(x_{ppp}/x_{ssp}\) ratios of immobilized peptides exposed to sugar solutions (peptide/sugar solution interface) are also shown in Figure 2a.

Figure 2b shows that as the \(x_{ppp}/x_{ssp}\) ratio increases, the immobilized peptide tilt angle also increases, demonstrating that peptides tend to lie down on surfaces at a high \(x_{ppp}/x_{ssp}\) ratio within the range 1.5 to 2.2. A \(x_{ppp}/x_{ssp}\) ratio lower than 1.5 is consistent with peptides exhibiting multiple orientations rather than one specific orientation.\(^{[44]}\) Figure 2a shows that when the sucrose concentration used for spin-coating was between 25 and 225 mM, the measured \(x_{ppp}/x_{ssp}\) ratio was smaller than 1.5. This shows that the immobilized peptides may adopt a multiple orientation distribution, e.g., they may have contributions from at least two different tilt angles. At a concentration of 225 mM, the tilt angle calculated was close to zero, indicating that the peptide was oriented along the surface normal. As the concentration increased from 225 to 275 mM, the peptides, which likely adopted a single orientation, increasingly tilted away from the surface normal. The peptides lies down when 275 mM sucrose was used for spin coating. Therefore, the tilt angle of the immobilized nMSI-78 peptide could be controlled by simply adjusting the sucrose solution concentration used for spin coating.

As described above, the different concentrations of the sugar solutions used for spin coating lead to different immobilized peptide orientations. Such orientation differences may be due to the different interactions between the immobilized peptides with sugar solutions with different concentrations. To study such possible differences, SFG spectra were collected from the surface immobilized peptides when exposed to sucrose solutions with different concentrations (Figure S4). Regardless of the sugar concentration, the SFG spectra are dominated by a signal centered at ~1650 cm\(^{-1}\), indicating the alpha-helical structure of immobilized peptides. Also, for all the sugar solution concentrations, the measured \(x_{ppp}/x_{ssp}\) ratio was around 1.70 (Figure 2a), which suggests that the immobilized nMSI-78 was standing up on the surface with a tilt angle of around 25°. This suggests that the
different orientations of immobilized peptides with sugar layer coated with different concentrations of the sugar solution in air are not due to the different interactions between peptides and sugar solutions with different concentrations. Such a difference must be induced in the spin coating process or the sugar solution drying process.

To further understand how the spin coating process or drying process affects the structure of the surface immobilized peptide, three different sugar “film-forming” methods were used to coat the sugar layer on peptide immobilized surfaces in air: spin coating, solvent casting with fast drying, and solvent coating with slow drying. SFG spectra were then collected from the surface immobilized peptides with sucrose coating prepared using the above three methods. The results obtained from these three methods (details of the procedures of the three methods can be found in the supporting information) are not the same, as shown in Figure 3. As Figure 1d showed previously, on the sucrose spin coated surface, peptides were ordered and generated strong SFG signals at 1650 cm$^{-1}$. Similar to the spin coating, when the solvent casting fast drying method was used to prepare the sugar layer, the alpha helical structure of the immobilized peptides was stabilized with a specific orientation (Figure 3d). In contrast, after coating a sucrose layer on the surface immobilized peptides using the solvent casting slow drying method, SFG amide I signal was not detected from the surface immobilized nMSI-78 in the air (Figure 3b). Apparently, during both the spin coating and solvent coating with fast drying processes, the surfaces were dried quickly – while the drying process was slow during the solvent casting with slow drying process. Both the “fast-drying” methods could retain the conformation and orientation of the underneath immobilized peptides. However, the solvent casting with slow drying method could not. Moreover, the sugar layers from both “fast-drying” methods clearly have different interactions with immobilized peptides from those from “slow-drying” method, shown in Figure 3b, which indicates that spin coating itself does not induce the orientation difference. Thicknesses of the sugar film prepared by spin coating with different sugar solution concentrations were measured (Figure S5). As the sugar solution concentration increases, the resulting sugar film thickness increases. We therefore believe that the sugar film thickness may influence the drying rate, which influences the peptide orientation. Likely, the sugar may form different amorphous/crystalline phases when drying at different speeds. We performed X-ray diffraction analysis on the sugar coating layer on immobilized peptides prepared with the above three different methods. No X-ray diffraction pattern was observed from any of the three sucrose coatings (data not shown here). Therefore, the possible role of different sugar amorphous/crystalline phases is still speculative. Another possible reason for the differences maybe because, during the drying process, the sugar molecules retain a small amount of bound water and form a glassy state. The different drying rates may lead to different bound water amounts and different sugar-water-peptide interactions. More details of the effect of sugar coating preparation methods on the immobilized peptide conformation/orientation are being studied.

Similar effects have been found and studied with protein using freeze drying method. Thermodynamic studies have found that with sugars in solvent, unfolding of a protein becomes thermodynamically less favorable.\textsuperscript{[11]} Other studies showed that sugars form strong hydrogen bonds with proteins, stabilizing the protein structure.\textsuperscript{[14, 40]} In addition, sugars can modify the hydration layer of a protein in aqueous buffer which eventually can reduce the flexibility of the protein, making it more rigid and stable.\textsuperscript{[46]}

In this work we studied how sucrose coating affects the secondary structure and the orientation of surface immobilized peptides at RT in dry conditions. First, we observed that spin coated sugar on top of the surface immobilized nMSI-78 at RT could support and orient the alpha-helical secondary structures of the immobilized peptides. The sucrose’s effect was observed to be very stable at even higher temperatures (Figure S6). Then, we studied the effect of the concentration of the sugar solution used for spin-coating on the immobilized peptide structure. It was found that the immobilized peptide orientation could be varied by varying the concentration of the sugar solutions used for spin-coating. Therefore, changing the sugar solution concentration allows us to easily control the orientation of the surface immobilized peptides, which may build a foundation for the control of conformation and orientation of more complex biomolecules such as enzymes in air for optimized sensing response in the future. This immobilized peptide orientation control method using spin-coating was also found to be reversible (Figure S7). We discovered that in order to retain the standing-up alpha-helical structure of immobilized MSI-78 on the surface, it is necessary to quickly dry the sugar molecules on the peptides to “freeze” the secondary structure of the peptide with a specific orientation. More details about the drying process and the effect of different drying rates are under the current investigation. Interestingly, it was found that spin coated sugars of different types, e.g., sucrose, trehalose and mannitol, have different influences on the conformation and orientation of immobilized peptides underneath, which are also under the current investigation.

In this study the nMSI-78 peptides were immobilized onto a self-assembled monolayer. In the future we will immobilize peptides/proteins onto more complex surfaces and use a variety of sugar molecules to control the immobilized peptide/protein conformation and orientation in air without the presence of bulk water. We believe that this will preserve the functions of biological interfaces in harsh conditions.

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Notes and references