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Sticky water surfaces: Helix–coil transitions suppressed in a cell-penetrating peptide at the air-water interface

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GALA is a 30 amino acid synthetic peptide consisting of a Glu-Ala-Leu-Ala repeat and is known to undergo a reversible structural transition from a disordered to an α-helical structure when changing the pH from basic to acidic values. In its helical state GALA can insert into and disintegrate lipid membranes. This effect has generated much interest in GALA as a candidate for pH triggered, targeted drug delivery. GALA also serves as a well-defined model system to understand cell penetration mechanisms and protein folding triggered by external stimuli. Structural transitions of GALA in solution have been studied extensively. However, cell penetration is an interfacial effect and potential biomedical applications of GALA would involve a variety of surfaces, e.g., nanoparticles, lipid membranes, tubing, and liquid-gas interfaces. Despite the apparent importance of interfaces in the functioning of GALA, the effect of surfaces on the reversible folding of GALA has not yet been studied. Here, we use sum frequency generation vibrational spectroscopy (SFG) to probe the structural response of GALA at the air-water interface and IR spectroscopy to follow GALA folding in bulk solution. We combine the SFG data with molecular dynamics simulations to obtain a molecular-level picture of the interaction of GALA with the air-water interface. Surprisingly, while the fully reversible structural transition was observed in solution, at the water-air interface, a large fraction of the GALA population remained helical at high pH. This “stickiness” of the air-water interface can be explained by the stabilizing interactions of hydrophobic leucine and alanine side chains with the water surface. © 2014 AIP Publishing LLC. [http://dx.doi.org/10.1063/1.4898711]

I. INTRODUCTION

Peptides can be used in drug delivery systems and enable drugs or drug-loaded particles to be delivered to specific locations within tissues or cells.1–6 The pH sensitive peptide GALA (WEAALAEALAEALAEALAEALAEALAA) is a mimic of viral fusion proteins and provides a promising route to achieve site-specific delivery of therapeutic compounds.1 The membrane penetrating activity of GALA is triggered by changes in the environmental pH. At basic pH, GALA assumes a disordered structure, but when lowering the pH to acidic conditions, the peptide transitions into an α-helical structure. In this state, GALA has the ability to penetrate cells by pore formation and membrane degradation. This mechanism is driven by the protonation and deprotonation of the Glu residues. The pH driven peptide activity is particularly interesting, because it can facilitate the escape of particle-encapsulated drugs from endosomes after endocytosis.7,8 In analogy to viral escape strategies, this approach makes use of pH differences between endosomes and the cytosol. The low pH environment in endosomes (pH ≈ 5.5) can activate GALA immobilized at drug-loaded particle surfaces and allows the particles to escape into the cytosol. The higher pH in the cytosol (pH ≈ 7) deactivates the peptides, rendering them harmless for other cell organelles.

GALA’s transition from random coil to an amphipilic α-helix roughly occurs when the pH is decreased from 7 to 5, the pH of inflection between the two conformational states was reported to be near pH 6.9–12 At low pHs the Glu sites are protonated and uncharged. The hydrophobic periodicity of the EALA repeats allows the peptide to fold into a stable amphipilic helix with hydrophobic leucine and alanine sites on one, and hydrophilic glutamic acids on the other side of the helix (Scheme 1). At neutral pH, however, charge-charge interactions between the deprotonated Glu side chains destabilize the helix fold. In the following, the protonated and deprotonated states of GALA will be denoted as GALA0 and GALA−, respectively. Note that this nomenclature only refers to the charges on the Glu sidechains, not to the total charge of the peptide, where the protonation state of the His sidechains and the termini would have to be accounted for.

Fourier-transform infrared (FTIR) and circular dichroism (CD) studies in solution show that in its helical state GALA

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self-associates into oligomers with the ability to penetrate the hydrophobic core of lipid membranes, form pores and induce leakage. Along those lines, multiple investigations of GALA as a vector for drug or gene delivery have been conducted. What remains to be clarified is how hydrophobic surfaces—for example, drug loaded polymeric nanoparticles, biochips, and biotechnical equipment—may influence GALA’s pH-driven helix-coil transitions. Folding (and aggregation) at the (hydrophobic) air-water interface has been investigated in the context of understanding the conformational equilibrium of several protein systems that are reportedly intrinsically disordered in solution, undergo transitions to helical structures when in contact with membranes or hydrophobic interfaces, but are also able to from β-sheet rich (amyloid) aggregates at higher protein concentrations. Examples include amyloid-beta-peptide, α-synuclein and LK peptides.

The aim of the present study is an improved understanding of how GALA interacts with aqueous-hydrophobic interfaces and how binding to such surfaces affects the folding of GALA. As the easiest accessible water-hydrophobic interface we have chosen the air-water interface for this investigation. We probed the pH-dependent secondary structure by a combination of surface sensitive vibrational sum frequency generation (SFG) spectroscopy, infrared spectroscopy, and molecular dynamics (MD) simulations of GALA in solution and at the air-water interface.

II. MATERIALS AND METHODS

For practical reasons, vibrational spectroscopy was performed in D₂O instead of water and we therefore follow deuteration instead of protonation. For the sake of simplicity we refer to the interface as air-water interface and we replace the term (de)deuteration by (de)protonation. The pH values of all D₂O solutions were adjusted using a H₂O-calibrated pH-meter. The direct reading of the pH-meter in D₂O solutions, referred to as pD, was used throughout this article. The relation between pD and pH is pH = pD + 0.44.19

A. Synthesis

All Fmoc-protected L-amino acids and preloaded resin (Fmoc-Gly-Wang resin, 100–200 mesh, low loaded 0.30 mmol·g⁻¹) for solid phase peptide synthesis were purchased by Novabiochem (Merck). The purity of the commercial amino acids was ≥98%. N-[(1H-benzotriazol-1-y1)(dimethylamino)methylene]-N-methyl-methanaminium hexafluoro-phosphate N-oxide (HBTU, Novabiochem), ethyl cyano-glyoxylate-2-oxide (Oxyma Pure, Merck, ≥98%), N,N-diisopropylethylamine (DIEA, Fluka, ≥98%), trifluoroacetic acid (TFA, Acros, 99%), triisopropylsilane (TIS, Alfa Aesar, 99%), N-methyl-2-pyrrolidone (NMP, BDH, 99%), piperazine (Merck, ≥99%), and all solvents were used as received.

The peptide sequences were prepared using standard solid-phase Fmoc chemistry with a microwave assisted automated peptide-synthesizer (Liberty, CEM). Tryptophan was used as Fmoc-L-Trp(Boc)-OH, Histidine as Fmoc-L-His(Trt)-OH, and Glutamic acid as Fmoc-L-Glu(OtBu)-OH. The parameters used for the coupling and deprotection steps are given below and relate to 0.1 mmol of peptide. Coupling was achieved under 300 s of microwave heating, with a temperature reaching and stabilized at 75°C after around 90 s, with Oxyma Pure as activator (5 equivalents), DIEA as base (10 equivalents), and amino acid (5 equivalents). Then a first deprotection stage of 30 s (temperature reaching around 50°C at the end) was followed by a second cycle of 180 s (temperature 75°C) with a 20 wt. % solution of piperazine in dimethyl formamide. The resin was washed 3 to 5 times between each coupling or deprotection step. Before cleavage the resin was dispersed in dichloromethane and filtered. Cleavage of peptide from the resin was performed using a mixture of TFA/TIS/H₂O (95%/2.5%/2.5%) for 6 h at ambient temperature. After filtration, the peptides were precipitated and centrifuged three times in cold diethyl ether, and dried over vacuum at 30°C. To exchange the TFA with DCI counterions, 10 mg of the GALA were solubilized in 20 μl deuterated 2,2,2-trifluoroethanol (TFE) with 40 μl of D₂O (pH = 2.3, DCI) while being placed in an ultrasonic bath for 15 min. To support the deuteration of the N–H groups of the peptide bonds the vial was heated up to 38°C in a temperature controlled shaker for 2 h. After deep-freezing the solution in liquid nitrogen the peptide was placed in a freeze-drying system. After one night of freeze drying the procedure was repeated three more times. The Glu side chains in the purified state can be considered fully deuterated yielding the state of GALA²⁰.

B. FTIR

The FTIR spectra were recorded on a Bruker Vertex 70 equipped with a DLaTGS detector in transmission mode. A transmission cell with CaF₂ windows and 10 μm spacers was used for all measurements. All spectra were recorded at room temperature. Each spectrum was averaged for 62 scans at a resolution of 2 cm⁻¹. As a background (BG) reference the CaF₂ cell containing buffer solution without GALA was used. 15 μl aliquots of a 2.2 GALA solution (0.5 M K₂HPO₄, 99%), piperazine (Merck, ≥99%), and all solvents were used as received.
\( pH = 3 \) and \( pH = 12 \) were used for each measurement. The sample chamber was purged with dry air for 10 min before every measurement. The software OPUS was used to display, record, and analyze the spectra. From all spectra we subtracted the spectrum of the BG recorded in the same measurement session since residual superposition of the amide bands with the broad D$_2$O bending mode band at 1555 cm$^{-1}$ did not allow comparing the spectra without this treatment. The exact peak positions were identified by the minimum positions in the second derivative spectra, which were calculated by means of the Savitzky-Golay algorithm (degree 2) allowing a concomitant smoothing (9 smoothing points). The amide I' region was fitted with a sum of profiles consisting of 50% Lorentzian shape and 50% Gaussian shape\(^{20}\) using the Levenberg-Marquardt algorithm.\(^{21}\) The resonance positions obtained from the second derivative spectra were fixed and the full width at half maximum (FWHM) as well as the band area were iterated. To check and further improve the quality of the fit we also compared the second derivative spectra of the fit with the original second derivative spectra. The resonance positions are reported with an error margin of \( \pm 2 \) cm$^{-1}$.

C. Tensiometry

Surface pressure curves were taken simultaneously with SFG measurements using a Kibron DeltaPi tensiometer, which was calibrated to air and the pure buffer solution first. The constant baseline measured at the beginning in absence of the peptide was set to zero.

D. SFG

For the SFG experiments we used broadband infrared pulses generated by an OPG/OPA (TOPAS, Light Conversion), which was pumped by \( \sim 1.7 \) W average power of 800 nm pulses from a Spitfire Ace (Spectra Physics) amplified laser system (1 kHz, \( \sim 40 \) fs FWHM). In addition, \( \sim 1 \) W of the laser output was branched off and directed through an etalon to generate the narrow band visible pulse (FWHM bandwidth of \( \sim 15 \) cm$^{-1}$, 20 \( \mu \)J). The broadband infrared pulse (\( \sim 3.5 \) \( \mu \)J) provided a 200 cm$^{-1}$ spectral window centered at 1700 cm$^{-1}$ in the amide I region. The visible and infrared beams were spatially and temporally overlapped at the solution surface. The incident angles of the visible (VIS) and infrared (IR) beam were \( \sim 35^\circ \) and \( \sim 40^\circ \) with respect to the surface normal. The VIS beam was focused down to a diameter of approximate 400 \( \mu \)m. The SFG light was spectrally dispersed by a monochromator and detected by an Electron-Multipled Charge Coupled Device (EMCCD, Andor Technologies).

A trough (70 \( \times \) 40 \( \times \) 5 mm) was filled with 4 ml D$_2$O containing 10 mM K$_2$HPO$_4$. The initial pH was set to 11–12. 20 \( \mu \)l of the same solution containing 0.14 mg of GALA\(^0\) was sonicated for twenty minutes and added to the subphase of the solution in the trough to achieve a final concentration of 9 \( \mu \)M, which is below the concentration at which self-association occurs in bulk solution.\(^{9}\) The samples were allowed to equilibrate until the surface pressure remained constant before the SFG measurements were performed. To fully protonate or deprotonate the Glu at the air-water interface we added a certain amount of DCI or NaOD to the subphase to yield pHs of 3 or 12, respectively. The amount of DCI and NaOD needed was determined independent of the SFG experiment using a pH-electrode.

The spectra were corrected for the background and normalized through a reference spectrum generated by the non-resonant SFG signal of a silver surface. Spectra were recorded in ssp (s-polarized SFG, VIS, and p-polarized IR beams) and sps polarization with 600 s of acquisition time. The background signal was also recorded with 600 s of acquisition time while blocking the IR pulse.

E. Molecular dynamics simulations

Two sizes of GALA peptides were studied by MD simulations: the full-length peptide consisting of 30 residues and a shorter version with only 16 residues (WEALAEALAEALAEH) corresponding to the first half of the repetitive sequence, from now on denoted as half-GALA. The latter was chosen to more extensively study the folding/unfolding equilibrium and the partitioning process between the bulk water phase and the air-water interface at different pH conditions, i.e., protonation states of the peptide. Simulations were performed with the GROMACS simulation package\(^{22}\) using the GROMOS 57a7 force field\(^{23}\) and the SPC water model.\(^{24}\) The peptide was solvated in a rectangular box for the simulations with an air-water interface or in a dodecahedral box for the bulk-solution simulations. The system was neutralized by sodium ions depending on the peptide charge (GALA\(^0\), GALA\(^{7-}\), half-GALA\(^0\), and half-GALA\(^{4-}\)). The initial box size was chosen such that the minimum distance between the box edge and the protein was larger than 1.5 nm. Non-bonded interactions were calculated with a twot-range cutoff scheme, with an update of the short range van der Waals and the real-space Coulomb interactions (\( < 1.0 \) nm) every time step, and an evaluation of the van der Waals interactions between 1.0 and 1.4 nm together with the neighbor list every 10 time steps. The long-range Coulomb interactions were calculated by the particle mesh ewald method\(^{25,26}\) with a grid spacing of 0.12 nm. All bonds were constrained by the LINCS algorithm\(^{27}\) and a time step of 2 fs was used. The solvated and neutralized systems were energy-minimized with position restraints on the protein atoms (1000 kJ mol$^{-1}$ nm$^{-2}$) and subsequently without restraints by the steepest descent and then by the conjugate gradient algorithm. In the next step, several 200 ps long equilibration simulations were performed with position restraints on a decreasing number of peptide atoms: initially all peptide atoms were restrained, then only the backbone atoms and finally only the alpha carbon atoms. During the equilibration phase the Bussi velocity rescale method\(^{28}\) was used to keep the temperature at 298 K (with a coupling time of \( \tau_p = 0.1 \) ps). The interface simulations were performed under constant volume conditions while for the bulk systems the pressure was maintained at 1 bar using the Berendsen weak coupling method\(^{29}\) with a coupling time of \( \tau_p = 0.5 \) ps. For the production simulations (total
length: 2000 ns for the half-GALA systems, and 100 ns for the full-length GALA systems) the temperature was maintained by the Langevin thermostat with a friction coefficient of 1 ps\(^{-1}\).

F. SFG spectra calculation

SFG spectra were calculated using methods described in Ref. 30. We obtained the relative spatial arrangement of the amide groups in the protein from the molecular dynamics simulation. From the coordinates we determined the couplings between the amide groups: nearest-neighbor couplings are calculated using an \textit{ab initio} map that gives the coupling as a function of the dihedral angle between the subsequent amide groups, and non-nearest neighbor couplings are calculated with a transition dipole coupling model. The local amide-I mode frequencies are shifted according to the hydrogen bonds that they participate in, and are also red shifted for amide bonds upstream of proline residues. After diagonalizing the Hamiltonian obtained in this way, we calculate the IR transition-dipole moments and Raman tensors of the amide I normal modes from the eigenvalues and eigenvectors, and we take their tensor product to calculate the vibrational SFG response.

III. RESULTS AND DISCUSSION

A. pH-driven refolding of solution state GALA

FTIR spectroscopy in transmission mode was used to verify that the GALA peptides used in this study are fully functional and show the expected helix-coil transition in solution. The FTIR spectra of GALA in its fully protonated and deprotonated state (recorded at pH = 3 and 12, respectively) are shown in Figure 1. All spectra exhibit an amide I band centered around 1650 cm\(^{-1}\) in the protonated (low pH) and 1644 cm\(^{-1}\) in the deprotonated (high pH) state (Table I). A band at 1540 cm\(^{-1}\) observed in the GALA0 spectrum corresponds to the amide II vibrational modes. Both spectra exhibit a band around 1440 cm\(^{-1}\). This band can be assigned to the amide II modes which are shifted by the deuteration of the backbone N–H groups. This indicates that only partial deuteration of N–H groups in the amide bonds of GALA occurs, as has been observed before for GALA in the helical state for which N–H appear to have extremely slow O–D exchange rates.\(^{13}\) However, the GALA\(^{-}\) spectrum does not exhibit any bands related to protonated N–H groups. This indicates that the main chain N–H groups are accessible to the surrounding water and therefore fully deuterated. The difference in the extent of backbone deuteration can be explained by a greater accessibility of the backbone atoms in the nonhelical state. A broad signal at 1706 cm\(^{-1}\) sensitive to pH occurs most distinctly in the GALA\(^{0}\) spectrum and can hence be assigned to the carbonyl stretching mode of protonated Glu.\(^{31}\) The GALA\(^{-}\) spectrum does not contain this band. Instead, two distinct additional bands sensitive to the deuteration state occur at 1568 and 1407 cm\(^{-1}\). These bands can be assigned to the asymmetric and symmetric carbonyl stretching vibrations of deprotonated Glu, respectively.\(^{31}\) The bands at 1568 and 1407 cm\(^{-1}\)—which can serve as valuable probes for the glutamic acid protonation state—were not observed in the attenuated total internal reflection FTIR spectra reported before.\(^{13}\)

A detailed view of the amide I region is shown in Fig. 1(b). The spectrum of GALA\(^{0}\) exhibits a strong band at 1653 cm\(^{-1}\) assigned to \(\alpha\)-helices, which accounts for 70% of the full band area. A band around 1630 cm\(^{-1}\) indicates \(\beta\)-sheet content accounting for 28%. The findings are in line with the previous study.\(^{13}\) The spectrum of GALA\(^{-}\) also contains an \(\alpha\)-helix contribution at 1653 cm\(^{-1}\) however accounting for only 27%. Similarly, the \(\beta\)-sheet content has significant lower contribution with 15%. On the other hand, a new

\[\text{FIG. 1. Helix-coil transition of GALA in solution. (a) Transmission FTIR spectra of GALA at pH 3 (red) and pH 12 (black). (b) Detailed view of the amide I region for the pH 3 and the pH 12 condition. The bands were fitted and assigned to \(\alpha\)-helices (pink), \(\beta\)-sheets (blue), random (green), and \(\beta\)-turns (orange).}\]
band at 1644 cm\(^{-1}\) indicates random structures and accounts for 53\%. From these results, we can confirm the majority of GALA molecules adopt random structures in the deprotonated state, whereas in the protonated state GALA adopts mostly \(\alpha\)-helical structures.

To obtain a detailed picture of pH induced helix-coil transitions of GALA in solution we have performed MD simulations of GALA in its protonated and deprotonated state. Due to the relatively large size of the full GALA peptide it is unfeasible to explore the full folding equilibrium at all different states of interest by MD simulations. We therefore decided to study half-GALA: a peptide consisting of the first 16 amino acids of the full sequence starting from the C-terminus (WEAALAEALAEALAEH). This allowed us to run longer simulations and reach timescales where folding/unfolding takes place and an assessment of the conformational equilibrium is accessible to molecular simulations. Due to the repetitive sequence of GALA, these simulations of half-GALA should permit conclusions on the conformational preferences of the full-length peptide, which will then be used to guide the interpretation of the IR and SFG data.

Figure 2(a) summarizes the results of the folding simulations of half-GALA in bulk aqueous solution. The left panel displays the evolution of the secondary structure as a function of simulation time and residue number, while on the right-hand side representative snapshots at different simulation times are shown. The first row corresponds to a simulation of half-GALA\(^0\) in the protonated state, which was started from an \(\alpha\)-helical structure. The core of the helix remained stable for most of the simulation time, with some unfolding and refolding occurring from the N-terminal side. After 1900 ns the helix unfolded entirely, and from this simulation alone it is not possible to draw conclusions on the thermodynamic stability of the \(\alpha\)-helix compared to other conformational states, this will be discussed in more details below. As a next step, half-GALA\(^{−}\) was simulated, i.e., the molecule in its charged/deprotonated state—again starting from an initially \(\alpha\)-helical structure, to mimic the effect of pH-induced unfolding (see Figure 2(a), second row). As expected, the helix unfolded rapidly, and a highly dynamic equilibrium of a multitude of structures was observed, including conformations with bends, \(\beta\)-sheets, but also transient partial \(\alpha\)-helical folds. From this ensemble of unfolded structures, one structure (at 400 ns, indicated by the black bar) was used to start another simulation in bulk aqueous solution—after reprotonating the Glu side chains. The results of this simulation, which mimics refolding induced by decreasing the pH, are shown in the third row of Figure 2(b). Within 1300 ns of simulation time the half-GALA\(^0\) molecule folded back into

<table>
<thead>
<tr>
<th>Band (cm(^{-1}))</th>
<th>HWHM (cm(^{-1}))</th>
<th>Percent full area</th>
<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td>GALA(^7)−</td>
<td>1630</td>
<td>17.8</td>
<td>15 (0.5)</td>
</tr>
<tr>
<td></td>
<td>1644</td>
<td>28.4</td>
<td>53 (1.6)</td>
</tr>
<tr>
<td></td>
<td>1653</td>
<td>28.9</td>
<td>27 (0.8)</td>
</tr>
<tr>
<td></td>
<td>1673</td>
<td>11.9</td>
<td>4 (0.1)</td>
</tr>
<tr>
<td>GALA(^0)</td>
<td>1630</td>
<td>29.8</td>
<td>28 (0.8)</td>
</tr>
<tr>
<td></td>
<td>1653</td>
<td>27.8</td>
<td>70 (2.1)</td>
</tr>
<tr>
<td></td>
<td>1674</td>
<td>10.1</td>
<td>2 (0.1)</td>
</tr>
</tbody>
</table>

\(^{a}\)The error margin for the band position is \(\pm 2\) cm\(^{-1}\). The experimental error of the peak areas is given in parentheses.
a predominantly $\alpha$-helical structure, transiently even the full $\alpha$-helix was observed. This simulation nicely complements that of half-GALA\textsuperscript{47}, which had been started from the helical structure (first row of Figure 2(a)). While the long “lifetime” of the $\alpha$-helix had already been indicative of a certain stability, the observation of refolding into the $\alpha$-helix after repARATION shows clearly that this is a stable, probably the most stable, secondary structural motif for the protonated GALA sequence. The helical fold is probably in equilibrium with other metastable states, as indicated by the unfolding event observed at the end of the simulation in the first row as well as by the formation of different partially formed helices in the simulation in the third row. These simulation data are in very good agreement with the results from the IR experiments of full-length GALA described above.

**B. At the air-water interface**

To study how the pH driven refolding of GALA is affected by the presence of a hydrophobic surface, we combined SFG experiments and MD simulations of GALA at the air-water interface. Figure 2(b) summarizes the MD simulations of half-GALA, again showing the secondary structure evolution as a function of time together with a few representative snapshots. The first row of Figure 2(b) shows the simulation of half-GALA\textsuperscript{0}, i.e., the molecule in its protonated state with uncharged Glu side chains, while the second row shows the simulation of half-GALA\textsuperscript{−}, i.e., the molecule in its deprotonated state with charged Glu side chains. Both simulations had been started from an unfolded conformation (the same conformation which had already been used in the simulation depicted in the third row of Figure 2(a)). In both cases, the half-GALA molecule rapidly moved to the air-water interface and remained there for the rest of the simulation time. In both cases, the molecule folded into $\alpha$-helices within roughly 1000 ns, and remained in the $\alpha$-helical state, irrespective of the protonation state of the side chains. Inspection of the obtained structures revealed that the hydrophobic Leu side chains are oriented towards the vapor phase while the hydrophilic Glu sites are pointing towards the water phase. Due to this partitioning of hydrophobic and hydrophilic residues, the helical conformation is stabilized, to an extent that even overcomes the charge repulsion between deprotonated Glu side chains, which drives the unfolding of the GALA helix that had been observed in the bulk solution case.

This—in its extent somewhat surprising—stabilization of the $\alpha$-helical state of GALA at a hydrophobic surface was examined experimentally using SFG spectroscopy at the air-water interface. In analogy to other vibrational spectroscopies, amide modes observed in SFG spectra can be used to identify secondary structure motives. However, the selection rules of SFG dictate that an SFG response will only be visible from an interface where inversion symmetry is broken.\textsuperscript{32, 33} As a result of these selection rules we can expect that any amide vibrational mode observed within the spectra will only originate from ordered secondary structures at the air-water interface. Unbound peptides in the water subphase—even if close to the surface—will not contribute to the signal.\textsuperscript{34} SFG has been successfully used to probe protein and peptide secondary structures in the past. In those SFG studies $\alpha$-helix,\textsuperscript{35–37} $\beta$-sheet,\textsuperscript{35, 37} $\beta$\textsubscript{10}-helix,\textsuperscript{38} and protein aggregates\textsuperscript{39–41} have been identified.

Figure 3(a) shows a scheme of the experimental setup. In parallel with the SFG measurements we recorded the surface pressure throughout the experiment to follow the interface activity of GALA. The surface tension data are shown in Figure 3(b) along with the times of GALA injection, acid and base titration as well as the time points of SFG spectra measurements. The surface tension data show that, after the addition of solubilized GALA\textsuperscript{7−} (at pH 12) to the subphase of D\textsubscript{2}O (at pH 12), the surface pressure increases and levels off after 160 min. The increase and saturation of surface tension indicates GALA\textsuperscript{7−} is surface active and forms a layer at the air-water interface. This result is in good agreement with the observation in the simulations that GALA peptides show a strong affinity to the surface. The amide I SFG spectrum recorded using the $sp$ polarization combination (s-polarized SFG, s-polarized visible, p-polarized IR) after GALA\textsuperscript{7−} injection (“a” in the surface tension curve) is shown in Figure 4. The spectrum consists of a resonance near 1645–1650 cm\textsuperscript{−1}, which can be attributed to $\alpha$-helices.\textsuperscript{34, 42, 43} In complete agreement with the MD simulations, charged GALA—disordered in solution state—binds to the air-water interface and folds into a helical structure.

After 200 min the pH was decreased to 3. The surface pressure increased and stabilized after 300 min (Figure 3, (b)).
state b)—most likely due to an increase of the surface density of GALA. The protonation of the Glu sites reduces the electrostatic repulsion between the peptides and allows a denser peptide packing. The SFG spectra recorded in this state (“b”) are very similar to the high pH spectra. The amide signal contains again a band near 1645–1650 cm$^{-1}$ is visible in the spectrum, which can be assigned either to protonated Glu side chains or the carboxyl group at the peptide C-terminus. Titrating NaOD and DCI into the sample cell to elevate and reduce the pH for a second time (times “c” and “d”) showed that both, the surface tension as well as the SFG spectra, at high and low solution pH are very reproducible. It should be noted that towards the last cycle there is a noticeable increase of the surface pressure compared with the earlier cycles—most likely due to progressive screening of the Glu charges by salt ions which allow denser packing of GALA at the surface.

While SFG spectra for the protonated and deprotonated states at the air-water interface look very similar—they are not identical. What is the structural basis for the subtle spectral differences? Are they related to the minor differences noticeable in the MD simulations of interfacial half-GALA? SFG spectra in theamide I region can be congested with spectral features related to different helices, sheets, turns, and random structures within an 80 cm$^{-1}$ spectral window, making it difficult to unequivocally assign spectral shapes to secondary structure. This problem can be avoided by directly comparing experimental SFG spectra to spectra calculated from structure files of MD simulation trajectories. To this end we performed simulations of the full-length GALA sequence at the air-water interface (Fig. 5) in both the protonated and the deprotonated state. From the simulations of half-GALA it was already found that the $\alpha$-helix is the stable conformation at the interface in both protonation states. Therefore, only comparatively short simulations (100 and 200 ns) starting from the helical state were performed to capture small structural differences and local fluctuations that are induced by the differences in the side chain repulsion due to protonation/deprotonation. Figure 5 shows that under both pH conditions the helical fold is conserved. The deprotonated species GALA$^7^-$ exhibits a slightly larger extent of unfolding of the helix at the first few residues at the N-terminus.

Theoretical SFG spectra of the obtained MD snapshots of the full GALA sequence at 100 ns (shown in Fig. 5) were calculated using the methods described in Ref. 30. Figure 6 summarizes both experimental and calculated spectra in $ssp$ and $sps$ polarization for high and low pH conditions. For both the $sps$ and the $ssp$ polarization combination the calculated spectra match the experimental data very well. Both the main peak near 1645 cm$^{-1}$, as well as the low energy shoulder near 1610 cm$^{-1}$ observed experimentally, are reproduced in the calculated spectra. There are some deviations at high frequencies, which are not surprising since the Glu side chain modes are not included in the calculation. The close match of experimental spectra and those calculated from the MD structures strongly suggest that the obtained simulations provide an accurate description of GALA folding at the air-water interface.

The most striking result, however, is that both experiments and simulation show a large population of $\alpha$-helices at the interface—irrespective of solution pH. The stabilization is most likely a result of the escape of hydrophobic Ala and Leu residues from the water phase and their desolvation in the binding geometry found for GALA at the air-water interface. While the hydrophobic sites hold the peptides in place, the strong pH-dependence of the Glu band near 1700 cm$^{-1}$ shows that the protonation and deprotonation, the driving force for helix-coil transitions in GALA, are fully reversible. The air-water interface dramatically changes the balance of hydrogen bonding, adhesion, and charge repulsion forces within GALA peptides and inhibits pH-driven helix-coil transitions present in bulk water.
IV. SUMMARY

While we can confirm the pH-driven helix-coil transitions of GALA peptides in bulk solution observed in previous studies, we find that GALA does not show a pH-dependent structural transition at the hydrophobic air-water interface. Together, SFG experiments and MD simulations demonstrate that the unfolding of the GALA sequence is inhibited at the interface, most likely due to adherence of the hydrophobic side chains of alanine and leucine to the air-water interface—water surfaces are “sticky” for GALA peptides. This result strongly suggests that the reliability of the folding mechanism of GALA—for example, in pharmaceutical applications—will be strongly affected by side chain-surface interactions. Our study demonstrates that interfaces can generally change the balance of forces involved in structural transitions in “smart peptides,” and that surface–protein interactions will have to be taken into account for the rational design of responsive biomedical surfaces.

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