Exploring the Hydration Water Character on Atomically Dislocated Surfaces by Surface Enhanced Raman Spectroscopy

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ABSTRACT: Hydration is ubiquitous in any kind of water–substance interaction such as in various interfacial and biological processes. Despite substantial progress made to date, however, still less explored is the hydration behavior on complex heterogeneous surfaces, such as the water surrounding the protein, which requires a platform that enables systematic investigation at the atomic scale. Here, we realized a heterogeneous self-assembled monolayer system that allows both controllable mixing with hydrophobic or hydrophilic groups and precise distance control of the functional carboxyl groups from the surface by methylene spacer groups. Using surface-enhanced Raman spectroscopy (SERS), we first demonstrated the hydrophobic (or hydrophilic) mixing ratio-dependent $pK_a$ variation of the carboxyl group. Interestingly, we observed a counterintuitive, non-monotonic behavior that a fractionally mixed hydrophobic group can induce significant enhancement of dielectric strength of the interfacial water. In particular, such a fractional mixing substantially decreases the amide coupling efficiency at the surface, as manifested by the corresponding $pK_a$ decrease. The SERS-based platform we demonstrated can be widely applied for atomically precise control and molecular-level characterization of hydration water on various heterogeneous surfaces of biological and industrial importance.

INTRODUCTION

Hydration is a ubiquitous phenomenon in which water surrounds a surface (or solute), underlying diverse areas including colloidal, biological, geological, and even industrial applications. Compared to other simple liquid molecules, water has such an unique hydrogen bonding character that it exhibits networked and collective motion over a relatively large length beyond the scale of an individual water molecule. In this regard, for the study of hydration, such surface water interaction has been examined in the way that takes into account the surface area (or solute size) along with consideration of its chemical property. However, even though substantial efforts for theoretical or simulational understanding of hydration have been performed up to now, proper experimental platforms are still rare for the study of surface hydration because most platforms have been limited only to solution systems. There is a high need for a solid experimental scheme that enables systematic and precise investigation of the surface hydration phenomena.

Surface enhanced Raman spectroscopy (SERS) is one of the highly sensitive techniques to probe surfaces and is widely used in various fields such as catalysis, electrochemistry, material science, and biochemical and environmental sensing. Despite its capability to identify and obtain molecular structural information for numerous chemical species, SERS has a weakness, in particular, when investigating the water molecule directly, due to its very low Raman cross section. Only a highly electrified situation in an ultrapurified environment has guaranteed the identification of Raman fingerprints of water, except for a recent experiment on the water confined in the nanomeniscus between packed nanoparticles at ambient conditions, which exhibited ice-VII-like structures. Moreover, until now, the hydration system has been analyzed only by special data processing such as the multivariate curve resolution (MCR) method, which is restricted to the solution system.

Meanwhile, an interesting water-related application of SERS has been reported to probe optically the local proton concentration. It was observed that the pH-responsive chemical group such as benzoic acid changes dramatically the total aromaticity of the molecular structure only by a subtle change of (de)protonation in the carboxyl group. Such a delicacy leads to a substantial change of the SERS spectrum, which can be used as an optical sensing platform of the water environment.

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In this study, we demonstrate a novel SERS-based strategy that investigates the surface hydration phenomena in a quantitative as well as qualitative way. We used modified pH-responsive benzoic acid analogues with their vertical positions controlled at atomic precision. We modulated the surface area of hydrophobicity (or hydrophilicity) by fractional

Figure 1. Experimental schematics for SERS investigation of surface hydration. (A) Schematic image of the in situ Raman set up for pH-titration experiments. Highly concentrated gold nanoparticles are adsorbed on the inner capillary wall via the preadsorbed APTMS layer, followed by treatment of heterogeneous SAM formation. (B) Schematic view of heterogeneous SAM on the gold nanoparticle surface, especially on the SERS-active hotspot region. (C) Photographic image of the gold nanoparticle-adsorbed capillary.

Figure 2. Raman spectroscopic pH-response of the carboxyl group. (A) pH-dependent surface-enhanced Raman scattering (SERS) spectrum of 4-mercapto(methyl) benzoic acid (4-MMBA, n = 1), showing that the peak at 1400 cm$^{-1}$ [$\nu$(COO$^-$)] is enhanced gradually with the increase of pH, while the one at 1700 cm$^{-1}$ [$\nu$(C=O)] decreases simultaneously. (B) pH-invariant SERS spectrum of 4-isopropyl benzenethiol (4-IBT) or 4-hydroxyl benzenethiol (4-HBT) that shows no prominent features in the spectral silence regions (red colored) where the carboxyl-related groups exhibit the distinct pH-induced change. (C) SERS spectrum in the mixture of 4-MMBA (n = 1) and 4-IBT, allowing one to find the correlation between the mixing ratio and pH-response of the system.
mixing of hydrophobic (or hydrophilic) self-assembled monolayers, whose mixing ratio was confirmed exactly by the SERS analysis. Moreover, by controlling precisely the relative vertical positions, we investigated systematically how such surface dislocation affects the global properties of hydration water and the chemical reactions occurring on such surfaces.

RESULTS AND DISCUSSION

Experimental Schematics for SERS Investigation of Surface Hydration. For reproducible and stable SERS measurements, we conducted all the experiments at an in situ configuration as shown in Figure 1A. Various pH buffer solutions were flowed through the capillary during SERS measurements while maintaining the laser spot invariant in order to avoid spot to spot variation. For reliable pH-titration results in SERS measurements, an unwanted photo (or plasmonic)-induced chemical reaction was avoided by irradiating the substantially low power laser (below 200 μW) on the highly SERS-active medium where gold nanoparticles were densely adsorbed inside the glass capillary. Figure 1B presents the schematic of an enlarged view of the hot spot area on the SERS medium, showing that mixing of hydrophobic or -philic group influences the hydration water behavior, which in turn changes the local pK\textsubscript{a} value of the nearby benzoic acid group. The vertical position control was realized by changing the number of inserted alkyl groups (n = 0, 1, 2) beneath the benzene ring of the benzoic acid.

Raman Spectroscopic pH-Response of Carboxyl Group. Figure 2 presents our experimental strategy for SERS analysis. Figure 2A shows the observation of spectroscopic pH-response behaviors (red-colored regions) of the 4-mercapto(methyl) benzoic acid (4-MMBA, n = 1). Although such pH-dependent spectral change in the benzoic group (4-mercapto benzoic acid, 4-MBA) has been well established in previous experiments and simulation works,\textsuperscript{17−24} for the first time, observed that regardless of the number of alkyl groups underneath the benzene ring, the three benzoic acid analogues [4-mercapto(methyl) benzoic acid (4-MMBA, n = 1) and 4-mercapto(ethyl) benzoic acid (4-MEBA, n = 2)] exhibit intrinsically very similar spectroscopic pH-response behaviors (see Figure S1), allowing that the benzoic acid group can solely function as an independent pH-response unit in our measurements. Figure 2C shows the simultaneously measured Raman signals of the two mixed groups, pH-responsive 4-MMBA (n = 1, Figure 2A) and pH-invariant 4-isopropyl benzenethiol (4-IBT, Figure 2B). We could hypothesize that such a mixed spectrum leads us to derive the correlation between the mixing ratio (from green regions) and the pH-response (from red regions) in this heterogeneous system by

Figure 3. Hydrophobicity variation at a similar vertical position. (A) pH-titrated SERS-intensity change of the peaks at 1400 cm\textsuperscript{-1} [\nu(COO\textsuperscript{-})] and 1700 cm\textsuperscript{-1} [\nu(C\equiv O)], and their weighed intensity sum for 4-MBA that realizes similar height between groups. The SERS intensity for each pH was taken as the average value of more than 20 Raman spectra, and the spot to spot variation was substantially negligible during all measurements, with the associated error bars almost unnecessary. (B) Normalized plot of the pH-titrated fraction of COO\textsuperscript{-}, which is defined as the relative intensity of \nu(COO\textsuperscript{-}) component, for hydrophobic mixing with 4-IBT. The pK\textsubscript{a} value of 4-MBA is determined at a half fraction of COO\textsuperscript{-} as indicated by the horizontal dashed line. (C) Normalized plot of pH-titrated fraction of COO\textsuperscript{-} for hydrophilic mixing with 4-HBT. (D) Plot of pK\textsubscript{a} and free energy in the 4-MBA-based mixing system versus mixing ratio. Here, the positive (negative) value corresponds to hydrophobic (hydrophilic) mixing, showing the general behavior that hydrophobic (hydrophilic) mixing increases (decreases) pK\textsubscript{a}.
Figure 4. Anomalous non-monotonic hydrophobicity induced by atomic dislocation. (A) Plot of \( pK_a \) and free energy measured in the 4-MMBA-based mixing system with dislocation of a single alkyl group. The results show a fast decrease of \( pK_a \) with an increase of hydrophilic mixing with 4-HBT (blue dots), while a substantially decreased \( pK_a \) regime (red colored ellipse) appears until a 15% ratio for hydrophobic mixing with 4-IBT. (B) The pH-titrated fraction of COO\(^-\) clearly exhibits the anomalous \( pK_a \) variation at the same mixing ratios. (C) The hydrophobic anomaly of atomically dislocated surface was tested by the amide coupling reaction that occurs between the carboxyl group of 4-MBA analogue and the amine group of 4-ABN. (D) Two alkyl groups dislocated 4-MEBA-based mixing system shows that while hydrophilic mixing with 4-HBT induces a gradual decrease of \( pK_a \), hydrophobic mixing with 4-IBT exhibits a similarly anomalous \( pK_a \) regime (red ellipse) but covering a more increased mixing ratio (up to ~26%) than in the 4-MMBA-based system. (E) Comparison of Raman spectra (taken at pH = 12) between pristine 4-MMBA (red) and 1.2% 4-IBT-mixed 4-MMBA (green), obtained at the points marked in panel A. It is clearly evident that a tiny small fraction of 4-IBT induces a dramatic change in the 4-MMBA spectrum, especially in the pH-responsive bands. (F) Amide coupling reaction was confirmed by the evolution of isolated nitrile stretching band (red area) in the SERS spectrum. (G) Coupling efficiency was estimated using the peak area of nitrile (CN) group, which agrees well with the behavior of the \( pK_a \) value of 4-MMBA in panel A.

Comparing the unique molecular fingerprints of vibrational bands. Notice that the red areas in Figure 2C correspond to the spectrally silent region in the 4-IBT [or 4-hydroxyl benzenethiol (4-HBT)] spectrum in Figure 2B.

Hydrophobicity Variation at Similar Vertical Position. Figure 3 shows the results obtained at similar vertical positions as realized by using the 4-MBA \((n=0)\) monolayer (see Figure S1a and the inset of Figure 3B or C). Figure 3A plots the SERS intensity versus pH for the peaks (integrated areas) at 1400 cm\(^{-1}\) [\(v(\text{COO}^-)\)] and 1700 cm\(^{-1}\) [\(v(\text{C}=\text{O})\)], as well as their sum. Interestingly, we observed the hydrophobicity-induced change of pH response: As the mixing ratio of 4-IBT increased, the fraction of COO\(^-\) decreased in all pH ranges, as indicated by the shift of titration curves toward high pH (from red to green in Figure 3B). Here, we note that in our experimental scheme we inherently could not keep the surface density of the carboxyl group constant since it is exchanged by a hydrophobic (or -philic) one, resulting in the decrease of surface density. So, in this case, one might be suspicious about the possibility of density decrease induced-\(pK_a\) shifts of the carboxyl group. However, according to the simulation result for the same molecule in the previous report which shows the same pH-response in a single molecule level,\(^{28}\) it is likely that the pH-response would not be dependent on the surface density.

Such a hydrophobic increase of \(pK_a\) can be understood as follows. Bulk water molecules have a dielectric constant intrinsically high enough that they effectively screen the charged species such as cation or anion, and so diminish its electrostatic interaction in the water medium. However, the presence of hydrophilic surface in water, as first reported by the early work of Shellman,\(^{25}\) was proposed to function as an electrostatic enforcer between oppositely charged ions. A more recent experiment\(^{26}\) further clarified that a nearby hydrophobic surface substantially reinforces electrostatic interaction due to the decreased dielectric constant of water.\(^{27}\) However, we should note some other simulation results,\(^{28,29}\) which claim that although the water density at a hydrophobic surface is lower than at a hydrophilic one, its dielectric contribution is higher at a hydrophobic surface if its parallel and perpendicular components are carefully considered together. In this regard, the conclusion in ref 27 standing in contrast to such simulation results can be reconciled with a reinterpretation of the data.\(^{30}\) Apart from such debates on the hydrophobic surface effect, in our system we nonetheless observe that the adjacent hydrophobic group affects the equilibrium between protonated carboxylic acid (COOH) and deprotonated carboxylate (COO\(^-\)) with proton (H\(^+\)). As the surface becomes more hydrophobic due to the exchanged isopropyl group, the equilibrium state is shifted toward the more protonated state (COOH), i.e., the noncharged state, as also indicated by the increase of \(pK_a\) (Figure 3B), which indicates that the electrostatic interaction between carboxylate and proton is more strengthened. In contrast, on the other hand, mixing with the hydrophilic group is observed to move the equilibrium state toward the deprotonated state. This opposite behavior is reflected by the shift of titration curves from high to low pH, or the decrease of \(pK_a\), as indicated by the red-to-blue shift in Figure 3C.
Combining the results of Figure 3B,C, we plot the pKₐ of 4-MBA versus mixing ratio in Figure 3D. Here, for comparison, we assigned the negative and positive values to hydrophilic and hydrophobic mixing, respectively. We note that since all Raman measurements were conducted at more than 20 different laser spots in a single pH condition and by using its averaged spectrum we plotted the fraction of COO- with respect to each pH value (Figure 3B,C). From these curves (fraction of COO- vs pH), we determined the pKₐ when the fraction of COO- is half. Thus, the plot in Figure 3D already contains the information of the averaged value. Moreover, when we performed different sets of measurements, its pKₐ values were highly reproducible, with the associated error bars almost unnecessary.

As shown, in general, hydrophobic (hydrophilic) mixing induces the increase (decrease) of pKₐ. Notice we observed that pKₐ starts to decrease below ∼30% mixing, while there appears a slight shoulder even at the hydrophilic mixing of 4-HBT (refer to Supporting Information). To quantify the chemical energetics associated with the effect of such hydrophobic (or hydrophilic) group on the water-mediated pKₐ change, we estimated the free energy change of the acid ionization reaction in our mixed system, using \( \Delta G = -RT \ln K_a \) (R is the gas constant, \( T = 298 \) K, and \( K_a = -\log K_a \)). For the total change of pKₐ of carboxyl group by 4.8 from ∼90% mixing by 4-HBT to +75% by 4-IBT (Figure 3D), the corresponding free energy change is 6 kcal/mol, which is 2.7 times higher than the hydrogen bond strength in liquid water (∼2.25 kcal/mol); we hypothesize that the surface-induced change of water property is substantial enough to modify the water-mediated chemical reactions.

**Anomalous Hydrophobicity Induced by Atomic Dislocation.** As a next step experiment, we constructed atomically dislocated systems by using 4-MMBA that introduces only a small height difference (∼0.15 nm) of a single alkyl group spacer because the carboxyl group still shows a very similar pH-response in all analogue molecules (Figure S1). We note that characterization of such a mixed SAM system including the effect of height difference and surface density has already been reported in previous works.\(^{26,31-34}\) Figure 4A shows the pKₐ plot measured in the 4-MMBA-based mixing system. Interestingly, compared to the 4-MBA-based one (Figure 3D), we observed that mixing with hydrophilic 4-HBT exhibits much a faster decrease of pKₐ with the mixing ratio (blue dots in Figure 4A). The results propose that the OH group that is just atomically (∼0.15 nm) farther away from carboxyl group (4-MMBA/4-HBT) affects hydrophilicity much more strongly than the more closely located OH group in the 4-MBA/4-HBT system. Surprisingly, on the other hand, for the case of hydrophobic mixing with 4-IBT, we observed the unexpected behavior of pKₐ variation (green dots in Figure 4A); that is, the appearance of the regime of substantially decreased the pKₐ value (red colored ellipse) until a +15% mixing ratio. Additionally, we note that regarding the contact angle issue, although not shown here, the contact angle experiment fundamentally has been used to measure, macroscopically, the hydrophobic property of the surface which has a three-phase contact line of air, water, and surface. However, in our experiments, helped by the in situ SERS technique (very small hot spot area of around a few nanometers) and mixed SAM strategy, we have made it possible to measure the very local, microscopic, hydrophobic behavior of the hydrated surface (without any air contact situation), which is presented by the change of pKₐ of the carboxyl group.

For more quantitative analysis, in Figure 4E, we compared the SERS spectra between the pristine 4-MMBA (red curve) and +1.2% 4-IBT mixture (green curve), taken at the values marked by “pristine” and “D” in Figure 4A, respectively. Here, the 1.2% mixing was confirmed by comparing the relative Raman intensities between 4-MMBA and 4-IBT (dashed box in Figure 4E, more details in Supporting Information). We emphasize the observation that such a tiny addition of 4-IBT induces a dramatic change that the intensity of the COO⁻ band increases by 20%, while the C=O band vanishes completely, which clearly evidences the occurrence of hydrophilicity (i.e., increase of the fraction of COO⁻) induced by hydrophobic mixing. These results should be understood (as verified in the next paragraph) by the role of the mediated water network, not by the direct effect such as the intermolecular perturbation in the SAM system. Notice that the pKₐ change of 0.56 at +1.2% mixing corresponds to the quite noticeable free energy change of ∼0.8 kcal/mol per 0.15 nm dislocation. Notice also that in our SERS measurements, we could suppose that the total Raman signal comes from multiple hot spots, each of which covers a circle of ∼10 nm diameter (78.54 nm² area).\(^{35}\) Given that the +1.2% fraction of mixed group covers ∼0.94 nm² and the dislocation height is 0.15 nm, the number of water molecules penetrated into the dislocated volume is estimated as ∼5 molecules per single hot spot. Considering the number of water molecules on the first surface layer is assumed to be ∼873 in a single hot spot, it is rather remarkable that only 0.6% of dislocated water can change the remaining 99.4% water by at least ∼0.8 kcal/mol. Therefore, such surprising anomaly should be driven via the change of water character within at least a few nanometers range of optical hot spot. The pH-dependent SERS spectra taken at other mixing ratios are presented in Figure S2.

To confirm the anomalous pKₐ behavior with respect to chemical reaction at the surfaces, we have tested the amide coupling reaction between carboxyl and amine groups, whose rate can be affected by the local pH environment (Figure 4C). The 4-aminobenzonitrile (4-ABN) molecule was chosen to confirm the coupling via the Raman spectral appearance (in the range between 2000 and 2400 cm⁻¹) of the isolated nitrile stretching band (yellow circle in Figure 4C), whose peak area was used to measure the coupling efficiency (red area in Figure 4F). Interestingly, comparing with the pristine state, we could observe the significantly lowered coupling efficiency at the surface state “D”, although just 1.2% of the hydrophobic group was mixed with the 4-MMBA SAM. Such lowered coupling is comparably observed at the state “O”, where the hydrophilic group was mixed, proving once again the anomalous hydration water behavior in atomically dislocated surfaces. Note that the blue points in Figure 4G (i.e., same pKₐ values from Figure 4A) seem to generally follow the coupling efficiency (red bars).

For the dislocation-dependence experiment, we conducted similar measurements in the two alkyl group-dislocated 4-MEBA-based mixing system that might exhibit a height difference of ∼0.15 × 2 = 0.3 nm from the carboxyl group. As shown in the pKₐ plot in Figure 4D, hydrophilic mixing with 4-HBT induces a gradual decrease of pKₐ (blue points), suggesting naturally that the OH group that is a little farther away from the carboxyl group in 4-MEBA/4-HBT (Figure 4D) has a weaker hydrophilic effect than the more closely located group in 4-MMBA/4-HBT (Figure 4A). On the other hand,
Figure 5. Generality of crossover in hydrophobic hydration. (A) Our mixed SAM platform for surface hydration water, characterized by dislocation depth \( d \) (in unit of nm) and hydrophobic coverage \( N \) (or mixing ratio). Hydrophobicity anomaly appears in the lower \( pK_a \) region with an increase of \( N \), followed by hydrophobic crossover to a higher \( pK_a \) region. (B) Hydrocarbon solute system for hydration shell in bulk water,\(^7\) characterized by alkyl chain length and temperature. An anomaly appears in the stronger hydrogen bond (HB) region with an increase of \( n \), followed by hydrophobic crossover to the weaker HB region. Notice the conceptual similarity between the two crossovers (yellow ellipses in A and B) to higher \( pK_a \) and weaker HB with an increase of hydrophobicity.

As shown in conceptual schemes in Figure 5B, they showed spectroscopically the hydrophobic crossover of the hydration-shell water structure, which was very sensitive to the alkyl chain length (\( n \)) with respect to the variation of temperature (\( T \)) (upper panel in Figure 5B). Specifically, while small hydrocarbon solutes (below 1 nm length, \( n < 4 \)) induced the hydrophobically strengthened hydration water in all ranges of \( T \), the longer chains produced an abrupt crossover with \( T \), from a more ordered (i.e., hydrogen bonded) to a more disordered structure.

In comparison, our system can be simply modeled as in Figure 5A (upper panel), where the percentile hydrophobic coverage (\( N \)) and dislocation depth (\( d \)) represent the hydrophobic mixing ratio of 4-IBT and vertically added alkyl chain length in all 4-MBA analogues’ molecular backbone, respectively. Notice that the increased hydrophobic mixing causes an enhanced exposure of the hydrophobic surface to water environment (i.e., surface hydration water, Figure 5A), and thus \( N \) can be physically comparable to \( n \) of solutes surrounded by water medium (i.e., hydration shell, Figure 5B).

Now, in order to compare closely with the anomaly of our \( pK_a \) plot, we reorganized all the data in ref 4 and plotted again specifically versus \( n \) at each \( T \), and found interestingly there indeed appeared the crossover (lower-panel in Figure 5B; see also Figure S5). In particular, after the crossover point (see the yellow ellipse), the hydration shell has a weaker hydrogen bond (HB) than bulk water, as indicated by the region with the dotted upper arrows that are the mirror images of the lower arrows. In our \( pK_a \) plot (lower-panel in Figure 5A), we observe the very similar hydrophobicity-induced crossover from lower to higher \( pK_a \), that is, the chemical equilibrium is shifted from a more deprotonated (\( \text{COO}^- \) and \( \text{H}^+ \)) to a more protonated (\( \text{COOH} \)) state, which results from the increased electrostatic interaction between \( \text{COO}^- \) and \( \text{H}^+ \). Therefore, our \( pK_a \) plot (Figure 5A) can be matched reasonably to the HB strength plot (Figure 5B). Moreover, we find that while the crossover point (\( n \)) of hydration shell increases with the decrease of hydrophobic mixing.

hydrophobic mixing with 4-IBT produces still prominent anomalous hydrophobicity similar to that of the 4-MMBA-based system, that is, the emergence of the decreased \( pK_a \) regime (red ellipse). Notice that such an anomaly exists for mixing ratios of up to \( \sim 26\% \), more extended than the 4-MMBA-based system (detailed pH-dependent SERS spectra taken at some representative mixing ratios are shown in Figure S3).

To further validate that the above hydrophobic group induced anomalous \( pK_a \) behavior, we carried out additional separate hydrophobic mixing experiments using the typical benzene-type molecules. Figure S4 shows the plots of \( pK_a \), of 4-MBA, 4-MMBA, and 4-MEBA when mixed with benzenethiol (BT) molecules, demonstrating that such anomalous regime (red ellipse) also appears in all systems when mixed with BT. In particular, we observe that such an anomalous regime seems to be widened in proportion to the relative vertical distance between hydrophobic and carboxyl groups in the \( pK_a \) plot (see also the yellow arrows in Figure S4).

**DISCUSSION**

Since there have been debates about the understanding on the hydrophobic effect on water behavior, in an effort to try to explain intuitively and qualitatively our nonlinear (non-monotonic) observation (anomalously lowered \( pK_a \) regime observed during hydrophobic mixing in dislocated system), we have taken the viewpoint from the work of Amotz’s group.\(^4\) In their work, they first observed the strengthening of hydrogen bonding of hydration water at short alkyl chains, while at a longer chain length (\( n \geq 4 \)) they observed the weakening of bonding comparing to that of bulk state of water, which is claimed as hydrophobic crossover (actually, this was predicted earlier by Chandler\(^1\)). In the very similar way, we have also observed such a crossover in our platform, showing the change from low \( pK_a \) to high \( pK_a \) of the carboxyl group during hydrophobic mixing.

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temperature ($T$), our crossover point ($N$) of surface hydration water increases with the increase of dislocation depth ($d$). This leads to the possibility of an analogous effect between $d$ and $T$ as follows: a more deeply dislocated surface prevents kinetically water from freely moving around in the water network, just as a low temperature prohibits thermodynamically the movement of water molecules.

On the other hand, as an alternative explanation for such an anomalous, low $pK_a$ value during hydrophobic mixing (Figure 4A, D), we once again note recent simulation works, which claim that the water at a hydrophobic surface is a better dielectric, meaning that its dielectric contribution is higher at a hydrophobic surface if its parallel and perpendicular components are carefully considered together. According to such an explanation, hydrophobic mixing-induced low $pK_a$ observed in our experiments, is a rather expected phenomenon, since the resulting enhanced dielectric contribution can induce the chemical equilibrium of carboxyl group to a more deprotonated (charged) state. Although these simulation results seem to nicely support our anomalous $pK_a$ value at an atomically dislocated system, we still need a proper explanation for the hydrophobic mixing-induced low $pK_a$ increase that is observed at a similar vertical position system, which may be done in a future work.

In conclusion, we have established that our system allows us to systematically investigate the water behavior at chemically and geometrically controlled heterogeneous surfaces with ultimate position control. Combined with the molecular fingerprinting sensitivity of the SERS technique, our approach can be straightforwardly employed to unfold the water property on more complex surfaces. For example, the protein surface is highly heterogeneous both chemically (due to various side groups present in amino acids) and geometrically (resulting from surfaces of sub-nanoscale curvatures), and the challenging experimental investigation of the surface effect on the surrounding water can be realized by our platform. As a demonstration, we employed the triply mixed system (4-MBBA, 4-IBT, and 4-HBT) and showed that each component was readily discriminated both quantitatively and qualitatively (see Figure S6). Our results can also provide the potential capability in real biological systems and engineer the highly diverse hydrophobic or hydrophilic microenvironment by single atomic-level manipulation of the molecular backbone. Examples include the understanding the fundamental difference between the two similar amino acids, Asp (aspartic acid) and Glu (glutamic acid), which are chemically equivalent except for a single side chain, and controlling atomically the relative vertical or lateral position with respect to the neighboring group (either hydrophobic or hydrophilic). In particular, given that numerous biochemical reactions occur in a pH-sensitive environment, the observed hydrophobic anomaly can be applied to open a new route to control precisely and selectively the surface-induced chemical reactions as well as to offer a better understanding of the effect of surface hydrophobicity not only on the protein but also on various biological components such as DNA, enzyme, and lipid membrane.

### METHODS

**Chemicals.** 4-Mercaptobenzoic acid (4-MBA, 99%), 4-isopropyl benzenethiol (4-IBT, 95%), 4-hydroxybenzenethiol (4-HBT, 97%), benzenethiol (BT, 98%), and 4- amino propyl trimethoxysilane (4-APTMS, 97%) were all obtained from Aldrich and used as received. 4-Mercaptomethyl benzoic acid (4-MMBA, 95%) was obtained from Absolute Chiral. 4-Mercaptoethyl benzoic acid (4-MEBA) was supplied by a customizing company Medigen (Daejeon in South Korea), and the full synthetic protocol is given in Supporting Information. All pH-titration experiments were conducted using the standard buffer solutions (Samchun Chemicals) and its mixture with NaOH, whose pH values were additionally confirmed by the electronic pH indicator. Deionized water (resistivity ≥18.2 MΩ·cm) was used for washing the capillary during measurements.

**Substrate.** Gold nanoparticles were synthesized by conventional citrate-reduction methods, and their size of ~60 nm was confirmed by UV–vis absorbance and TEM analysis. We prepared a highly concentrated (more than 20 times) nanoparticle solution using a centrifugation process, and then adsorbed it onto the inner wall of conventional glass capillary (inner diameter 1.1 ± 0.2 mm, thickness 0.2 ± 0.02 mm, Kimble Chase) which was already prepared by thorough cleansing by piranha solution and subsequent amine-functionalization with 4-APTMS (using 1% ethanolic solution). To fabricate mixed SAM surfaces, 1 mM of ethanolic solutions for all the chemicals mentioned above were prepared as a stock and then mixed with them by balancing the volume ratio to control the relative molar ratio, while keeping the same volume (10 mL) for each mixing ratio. The gold nanoparticle-adsorbed glass capillary was dipped into the prepared SAM solution overnight to form the stable SAM on the nanoparticle surface and then thoroughly washed with ethanol. Buffer solution was injected using a syringe through the capillary tube, and the flow was controlled by the commercial syringe pump (NE-300, NEWERA).

**Instrumentation.** Raman measurements were conducted using our homemade system, consisting of an excitation laser (633 nm, He–Ne laser, Lasos), spectrometer (600 gr/mm, Monora320i, Dongwoo), and EMCCD (Newton, Andor). In order to avoid photobleaching or unwanted photochemical reaction, we used low laser power (<0.2 mW) with a short integration time (<10 s) and averaged the signals measured at various spots (more than 20 spots). In these experimental conditions, we also confirmed that the spot to spot or sample to sample signal variation was substantially low during all measurements.

### ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c01009.

Synthesis of 4-mercapto(ethyl) benzoic acid. Discussion on the shoulder of $pK_a$ during hydrophobic mixing. Comparison of intrinsic SERS intensity of each component. Figures S1–S6 (PDF)

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