

Peptides display multilayered hydration shell substructures dictated by sequence-specific surface chemistry

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Water is crucial for life, mediating intermolecular interactions that govern protein stability, molecular recognition, and biological function. While insights into protein hydration have been obtained using spectroscopic, scattering, and computational approaches, direct real-space visualization of hydration architectures at biomolecular interfaces with submolecular resolution remains challenging. Here we show that three-dimensional atomic force microscopy directly maps hydration architectures of self-assembled peptide molecular arrays formed on atomically flat graphite. We observe that a graphite-binding dodecapeptide, which mimics structural and chemical characteristics of proteins, organizes water into a multilayered, structured hydration shell. The structured water in the hydration shell exhibits a gradient of molecular ordering that is modulated by sequence-encoded surface chemistry, giving rise to domains of enhanced and diminished water organization that extend across hydration layers before gradually converging into the bulk water. The hydration structure dynamically conforms to the physical topography and the chemical domains of the peptides, with local water structure modulated by hydrophobic, polar, and charged peptide residues. Our findings reconceptualize hydration as a sequence-specific structural phenomenon, defining an integrated protein superstructure that incorporates the amino acid framework and its structured hydration shell, thereby providing a basis for understanding hydration-governed protein function and for designing proteins with tailored functionalities

Solid surfaces or molecules in aqueous environments are enveloped by hydration shells whose structure differs from that of bulk water due to the interaction of water molecules with the specific chemical, physical, and electrostatic characteristics of the surface^{1–7}. While crystalline inorganic solids with well-defined atomic lattices may exhibit relatively simple hydration structures, commensurate with their atomic and

ionic character^{4,8,9}, proteins are expected to possess more complex and highly structured hydration shells because of their intricate molecular folding, diverse chemical domains, and heterogeneous topological features incorporating neutral, charged, and polar amino acid moieties^{10–15}. The interplay of proteins with water may thus entail significantly intricate interactions due to the structural heterogeneity,

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conformational flexibility, and complex dynamics^{14–17}. These hydration-mediated interactions—arising from chemical diversity and conformational dynamics—are fundamental to protein functions, including folding^{10,14,15,17}, ligand binding¹⁸, molecular recognition^{19–23}, enzymatic activity²⁴, and proton transfer^{25,26}. In particular, the protein folding pathway and the resulting three-dimensional (3D) structural organization, characterized by a tightly packed hydrophobic core, are critically governed by hydration forces mediated through water-protein interactions^{12,14}. These interactions establish hydrogen-bond (H-Bond) networks around proteins that stabilize secondary and tertiary structures^{1,6,12,27}. Water-protein interactions play an especially prominent role in crowded cellular environments, where a substantial fraction (30%–70%) of intracellular water is influenced by proximity to biomolecular surfaces and contributes to the formation of hydration shells around biomolecules^{12,28,29}. The extent to which biomolecular surfaces perturb the structure of the surrounding solvent has long been a subject of investigation and is frequently discussed in terms of “biological water”. However, the existence of biological water as a distinct thermodynamic state remains controversial³⁰. Hydration water is generally regarded as an interfacial solvent whose local structure and dynamics are governed by the chemical heterogeneity, topography, and conformational fluctuations of the biomolecular surface. It has also been well realized that the extent and spatial organization of the hydration shells could critically influence protein-protein and protein-macromolecule interactions, and their structural stability in aqueous media¹¹. Despite their recognized importance and extensive prior study, a full understanding of the 3D molecular-scale architecture and spatial heterogeneity of protein hydration shells remains incomplete, constraining molecular-level insight into protein behavior, assembly, and rational design with predictive functions.

The polar nature of water enables highly directional intermolecular interactions—including charge-dipole and hydrogen bonding—with other water molecules and surfaces. In proteins, water facilitates non-covalent bonding through H-bonds at multiple sites, enabling highly specific ligand-receptor interactions^{14,27,31–33}. As a universal solvent, it envelopes polar and charged protein moieties^{34,35}, penetrates interstitial gaps, and modulates molecular bond strength—either stabilizing or destabilizing interactions^{36–38}. Traditionally, hydrated biomolecules were thought not to approach closer than $\approx 5\text{--}6\text{ \AA}$, corresponding to the thickness of two water molecules^{27,39}. However, recent studies have reported conflicting hydration shell structures, often due to limitations in experimental techniques and assumptions in computational models⁴⁰.

Resolving the real-space architecture of hydration motifs associated with specific sequence domains or surface features of proteins have been experimentally challenging. While significant progress has been made in characterizing biomolecular hydration, questions still remain regarding the extent of structural variations, molecular ordering, and spatial heterogeneity within hydration layers, particularly beyond the first hydration shell^{15,33,41}. A broad range of experimental^{15,31,34,36} and computational modeling studies^{42,43} have advanced our understanding of hydration structures around amino acid residues in folded proteins. In particular, spectroscopic and scattering techniques such as nuclear magnetic resonance^{44,45}, quasi-elastic neutron scattering^{33,46,47}, dynamic nuclear polarization⁴⁸, and 2D infrared spectroscopy^{49,50} have provided detailed insights into the structure and dynamics of hydration water in biomolecular and cellular environments. These approaches offer high temporal resolution and sensitivity to local environments, enabling characterization of hydration dynamics across multiple length and time scales. However, many of these methods probe hydration indirectly or rely on ensemble-averaged data, which can limit direct access to site-specific 3D organization of water molecules at sub-nm resolution at local positions. Recent studies generally support highly localized perturbations^{39,51,52}, whereas others report more extended structural or

dynamical effects^{13,53}. These contrasting observations underscore the complexity of biomolecular hydration and continue to stimulate efforts toward a more comprehensive understanding of its spatial extent and structural organization. Bridging the knowledge gap is fundamental for understanding how hydration stabilizes protein structures, guides folding, and mediates molecular recognition—the key to protein engineering and drug design. Resolving these questions experimentally remains challenging because protein interfaces are intrinsically heterogeneous, both chemically and physically, and conformationally dynamic. Such complexity induces rapid spatial and temporal averaging of hydration patterns, even under surface- or bulk-immobilized conditions, concealing confined hydration architectures that are inherently residue-specific and spatially localized from direct observation. Overcoming these challenges with high spatial and temporal resolutions, requires a bottom-up experimental strategy that isolates the contribution of surface chemistry while minimizing the effects of large-scale conformational fluctuations. Rather than attempting to image hydration directly at fluctuating protein interfaces, chemically defined, structurally ordered model systems offer a tractable route to stabilize hydration motifs and make them experimentally accessible.

Here we used a solid-binding peptide (SBP)⁵⁴, GrBP5-WT, that has a capability to self-assemble on atomically flat graphite⁵⁵, forming stable, long-range ordered, single molecule-thick peptide crystals with well-defined lattice architectures. Importantly, GrBP5-WT provides a structurally simple yet functionally rich model system that recapitulates key features of protein interfaces, including chemical heterogeneity and conformational diversity. The spatial organization of hydrophobic, polar, aromatic, and charged residues creates a diverse interfacial chemical landscape that captures essential aspects of protein surface complexity while remaining experimentally tractable (Fig. 1a–d). The molecularly flat topology enables high-resolution imaging in liquid by scanning probe microscopy^{56–58}. To directly probe hydration organization at these interfaces, we employed 3D atomic force microscopy (3D-AFM), which enables the visualization of local water structuring with sub-nanometer spatial resolution^{59,60}, thereby allowing characterization of hydration and solvation architectures on the peptide assemblies (Fig. 1b, c). As we discuss herein, this integrated approach reveals highly structured, spatially intricate hydration architectures surrounding chemically distinct peptide domains and provides a quantitative assessment of hydration shell thickness and sub-structural organization. Here, individual peptides are resolved using large-amplitude 2D frequency-modulated AFM (FM-AFM)⁶¹, while the hydration substructures are captured using small-amplitude 3D FM-AFM^{59,62}, yielding $\text{\AA}$ -thick hydration slices with high spatial resolution in all three dimensions (Fig. 1a, inset). The methodology and analyses presented here, particularly the direct observation of intricate peptide hydration architectures, provide a foundational framework for future investigations into protein structure, function, molecular recognition, and biomolecular interactions.

Results

Genetically selected SBPs with specific affinity for a given inorganic surface have recently been shown to form long-range ordered 2D molecular patterns with well-defined lattice structures that exclusively recognize the atomic lattice of their target inorganic crystals^{54,55,57}. Here, we used a directed evolution selected dodecapeptide, GrBP5-WT⁵⁵, with affinity to graphite surface (Fig. 2a–f). The molecular organization of GrBP5-WT is driven by highly selective, non-covalent peptide-peptide and peptide-graphite interactions, while recognition of the underlying carbon lattice is mediated by the surface-bound conformation of the peptide, resulting in templated nucleation and oriented planar growth^{55,58}. Consequently, GrBP5-WT peptides form a molecule-thick, 2D lattice on (0001)-oriented graphite, establishing an exclusive crystallographic chiral relationship

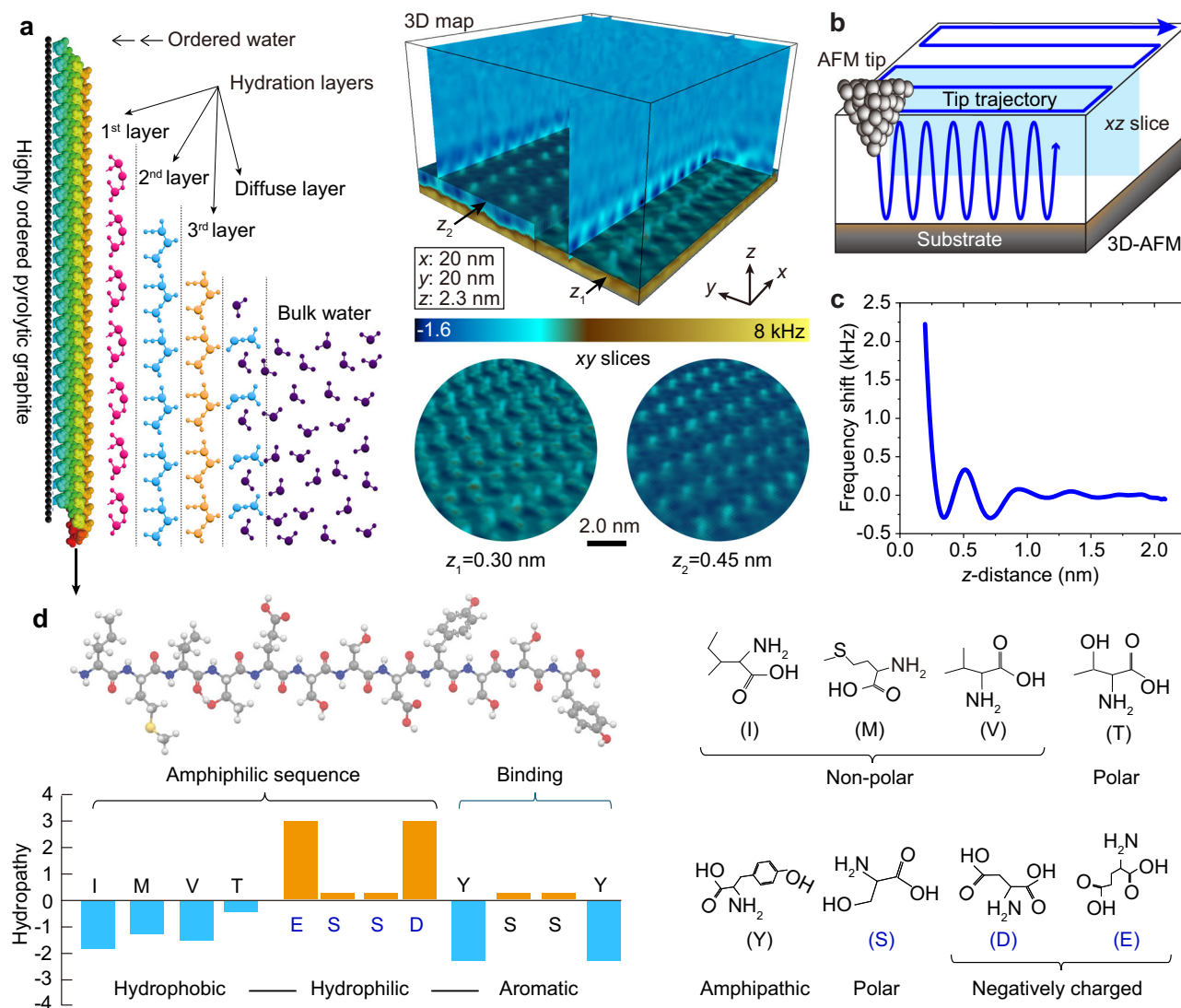


Fig. 1 | Molecular structure and 3D-AFM visualization of self-assembled dodecapeptide hydration shells. **a** Schematic illustration of water organization adjacent to the peptide assemblies, highlighting the formation of highly ordered hydration structures within the interfacial hydration shell. **b** Schematic of 3D-AFM applied to self-assembled peptide nanostructures on HOPG, showing extraction of 2D *xz* and *xy* slices from the reconstructed 3D volume map. The probe performs synchronized lateral (*xy*) and vertical (*z*) scanning, with a fast sinusoidal modulation superimposed on the *z*-position during image acquisition and force mapping. As the probe approaches the surface, characteristic oscillatory force profiles appear

c, reflecting discrete, ordered hydration layers that are progressively penetrated near the self-assembled peptide structures. **d** Extended molecular structure of the GrBP5-WT peptide shown in stick-ball representation alongside its amino acid sequence. The hydropathy map reveals three chemically distinct domains within the peptide. The inset in panel **a** presents a 3D-AFM volumetric map showing the molecular-resolution spatial organization of interfacial water surrounding peptide assemblies, including in-plane (*xy*) and cross-sectional (*xz*) views. Source data are provided as a Source Data file.

between the peptide molecular crystal and the underlying atomic graphite lattice (Fig. 2e). The formation of the peptide-graphite bio-nano soft interface is analogous to protein self-assembly processes involved in molecular recognition under physiological conditions^{63–65}, where structural organization and stability are governed by non-covalent interactions, such as electrostatic (Coulombic) forces, hydrogen bonding, and steric effects.

The molecular architecture and interfacial properties of the peptide assemblies were investigated in both ultrapure water and PBS buffer, revealing how ionic composition may modulate their structural organization and interfacial stability on graphite (Supplementary Figs. 1–5). The fast Fourier transform (FFT)-filtered AFM image obtained from the framed region in Fig. 2c, acquired in the PBS buffer, is shown in Fig. 2d, whereas the corresponding image obtained in water is shown in Fig. 2f. These observations provide molecular-level insight into the organization of the peptide assemblies and reveal the

emergence of ring- and ellipsoid-like structural features. High-resolution FM-AFM imaging at sub-molecular resolution further identified an oblique molecular lattice in both water and buffered environments, with unit cell parameters of $a = 3.2 \pm 0.2$ nm, $b = 1.6 \pm 0.1$ nm, and $\alpha = 72^\circ \pm 6^\circ$, as quantitatively summarized in Supplementary Fig. 6. The reproducibility of the lattice parameters across different solution conditions is consistent with our previous observation that GrBP5 forms the same ordered assembly on HOPG under comparable experimental conditions³⁸, demonstrating that peptide self-assembly is robust and largely insensitive to moderate variations in ionic composition. Importantly, the peptide molecular crystals with oblique symmetry maintain a persistent and highly selective chiral registry with the underlying graphite hexagonal C-lattice through epitaxial coupling (Fig. 2e). This registry arises from sequence-specific interactions between aromatic peptide residues and the C-lattice, which play a dominant role in surface anchoring and assembly orientation.

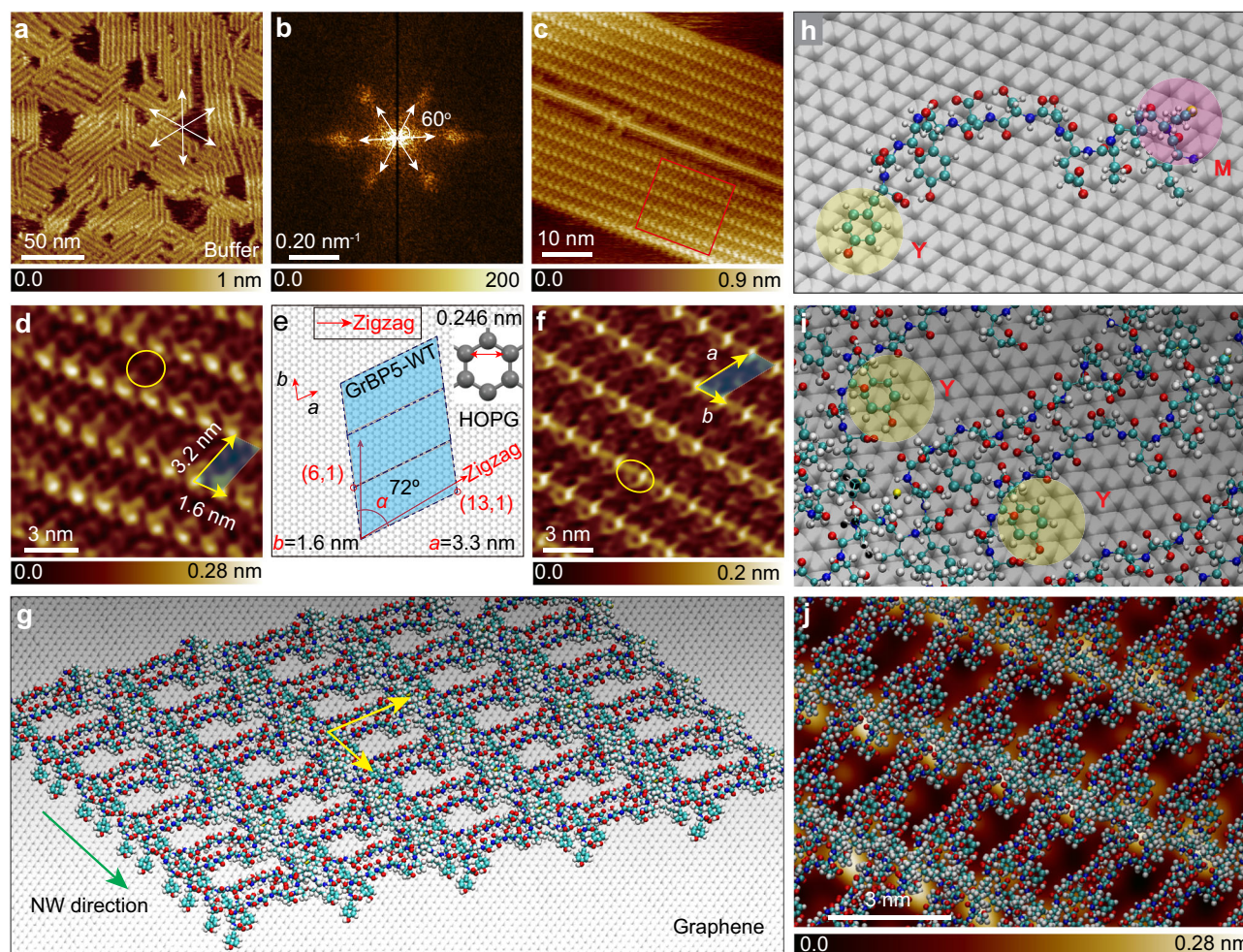


Fig. 2 | Supramolecular assemblies of GrBP5-WT peptides on HOPG in aqueous environment. **a** An overview AFM topography image of peptide nanostructure acquired in PBS buffer, showing long-range order with six-fold symmetric domains that are commensurate with the graphite lattice and **b** the corresponding 2D-FFT pattern confirming six-fold symmetry. The color scale represents the FFT intensity in arbitrary units. **c, d** Molecularly resolved AFM images of a stable peptide domain, acquired in PBS buffer, with the panel **d** showing a zoomed-in view of the framed region. **e** Crystallographic orientation relationships between peptide crystals and

the substrate lattice, defined by matching the peptide molecular lattice to the atomic lattice of the solid, enabling chiral recognition. **f** Molecular-resolution AFM image of a peptide domain in water. **g** Simulated structural organization of GrBP5-WT peptide assemblies on graphene. **h, i** Simulated molecular details show planar alignment of tyrosine (Y) residues in the binding domain enabling π - π stacking with the graphene lattice, while the methionine (M) residue in the IMVT domain protrudes above the surface, enhancing aqueous exposure. **j** Overlay of the simulated peptide assembly structure onto the AFM image in panel **d**.

We performed atomistic simulations using neural network potentials (NNPs) to elucidate the long-range molecular organization of the assemblies and identify the key interactions governing their stability, as described in the Methods section. Briefly, unit cells containing two GrBP5-WT molecules were identified as the most energetically favorable configurations, closely matching the experimentally determined lattice parameters. These molecular organizations also exhibited strong agreement with the experimentally observed peptide topography and yielded a physically plausible residue-level arrangement, as supported by the close correspondence between the simulated structures and AFM images (Fig. 2g–j and Supplementary Fig. 7). Within this assembled molecular structure, tyrosine residues adopt a planar orientation relative to the graphene lattice (Fig. 2i, j), facilitating π - π stacking interactions with the underlying C-lattice. In contrast, the methionine (M) residue within the IMVT hydrophobic domain protrudes above the graphene plane, occupying a topographically elevated position that is exposed to the surrounding solution. The central hydrophilic ESSD domain, enriched in polar and charged residues, acts as an electrostatically active region that counterbalances hydrophobic interactions, stabilizing and organizing the peptide assemblies

through hydrogen bonding and electrostatic interactions with water molecules and neighboring peptides. These results highlight the synergistic interplay of π - π stacking, hydrophobic interactions, and domain-specific electrostatic interactions, which collectively govern the ordered supramolecular assembly of the peptides and stabilize their interfacial adsorption. The resulting molecular lattice forms a structurally robust and chemically heterogeneous interface, in which amino acid side chains adopt reproducible spatial distributions while retaining local conformational flexibility. This reduction in configurational freedom is essential for maintaining long-range crystalline order and establishes a well-defined molecular landscape for interrogating interfacial hydration. By suppressing large-scale structural fluctuations while preserving chemically distinct functional groups, the peptide arrays provide an ideal platform for resolving hydration architectures with high spatial resolution.

The sub-nm resolution achieved in the FM-AFM images (Fig. 2d, f) facilitates detailed interrogation of salient sub-structural features within the folded peptide assemblies on graphite. In particular, the ellipsoidal and circular motif highlighted in Fig. 2f is frequently observed across multiple imaging sessions. The images are thought to

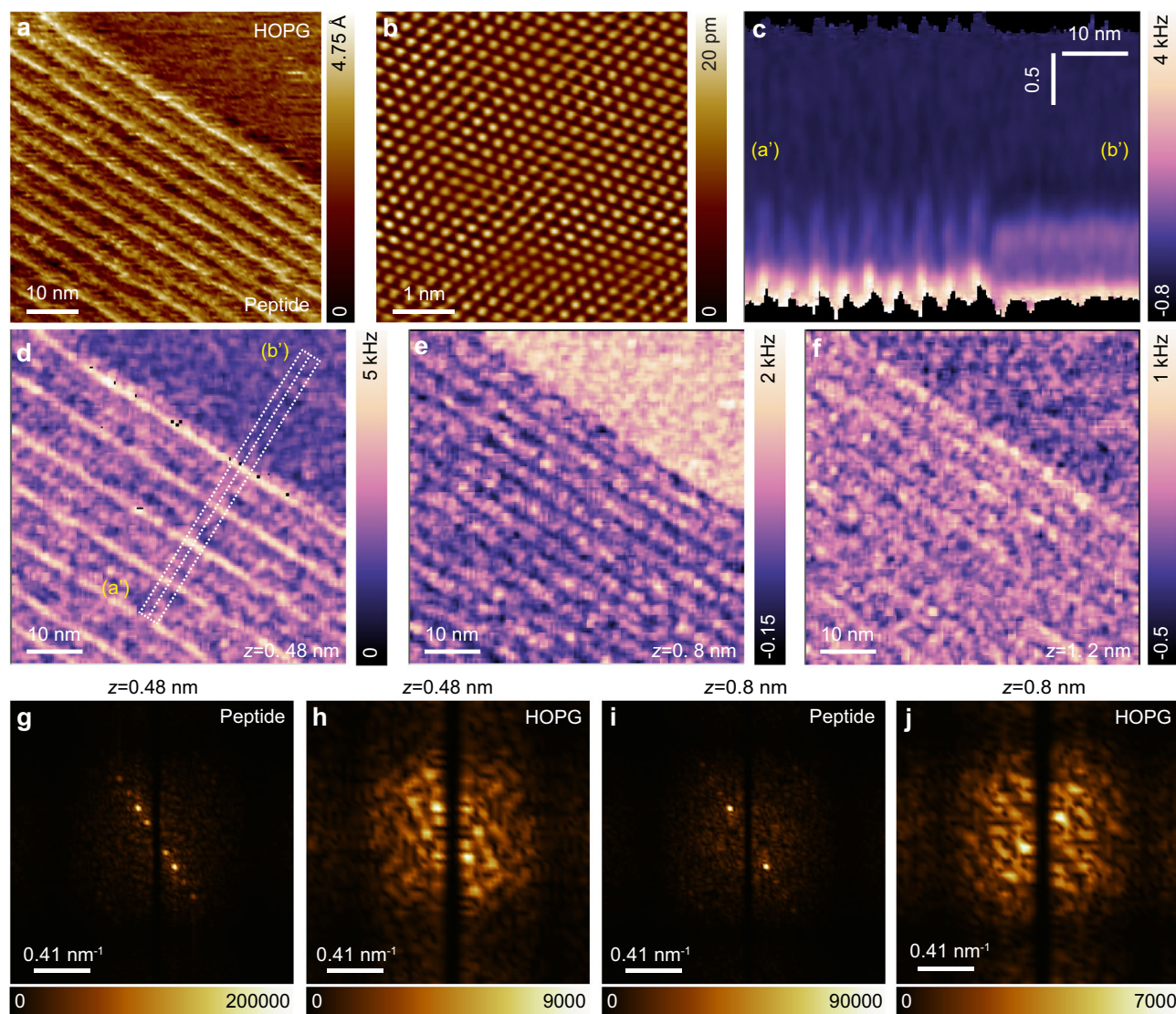


Fig. 3 | Molecular peptide assembly on a partially covered graphite surface enables simultaneous visualization of interfacial hydration structures in PBS. **a** 2D-AFM topography of self-assembled peptides on partially covered HOPG surface. **b** Surface lattice structural image of (0001) graphite. **c** Vertical xz slice extracted by averaging over the dashed rectangular region extending from a' to b' in **d**. **d–f** Lateral xy slices at selected z-distances, revealing in-plane water

structuring on peptides and HOPG. **g–j** FFT patterns obtained from peptide and HOPG domains at z = 0.48 and 0.8 nm show continuous hydration structures with lateral ordering on peptides, in contrast to discrete hydration layers lacking lateral molecular organization on HOPG. The color scale indicates the relative FFT intensity in arbitrary units.

arise from the complex interplay between the oscillating AFM tip and the combined physicochemical characteristics of the surface-bound peptides and their associated hydration layers. While the surface topography is a dominant contributor to image contrast, the assemblies also contain spatially and chemically distinct functional side chains and proximal H-bond networks. Consequently, the observed contrast features likely reflect localized interactions among the AFM tip, interfacial water molecules, and the three chemically distinct domains, as well as the spatially distributed and conformationally adapted physical structures of the peptides.

Submolecular 3D hydration mapping of peptide assemblies

The 3D-AFM measurements were conducted on graphite surfaces that are partially covered with peptide assemblies, enabling direct comparison of hydration characteristics between the peptide-covered and the bare graphite regions under identical acquisition and tip conditions (Supplementary Note 1). Hydration shells on graphite typically exhibit uniform layering with ≈ 0.5 nm interlayer

spacing in the z-direction, accompanied by a distinct density depletion near the interface, and lacking lateral molecular organization⁸, as shown in Fig. 3. A markedly distinct hydration profile was observed over the peptide-covered regions, characterized by the absence of a near-surface density depletion layer and the presence of laterally modulated hydration features extending into the aqueous phase. These observations indicate that the peptide crystals generate a complex multilayered hydration shell with nuanced substructures that progressively relax toward the bulk aqueous phase (Fig. 3; Supplementary Fig. 8). This complexity is consistent with the heterogeneous chemical and topographical nature of the peptide surface, which is expected to influence local water organization and dynamics. A sharp and well-defined boundary delineates the hydration layers of the peptide assemblies from those of the graphite (Fig. 3c–f). The observed hydration architecture is characterized by three characteristic features (see details in Fig. 4): (1) Long-range ordered hydration motifs extend throughout the 3D water column above the peptide lattice; (2) Nanoscale substructural features within

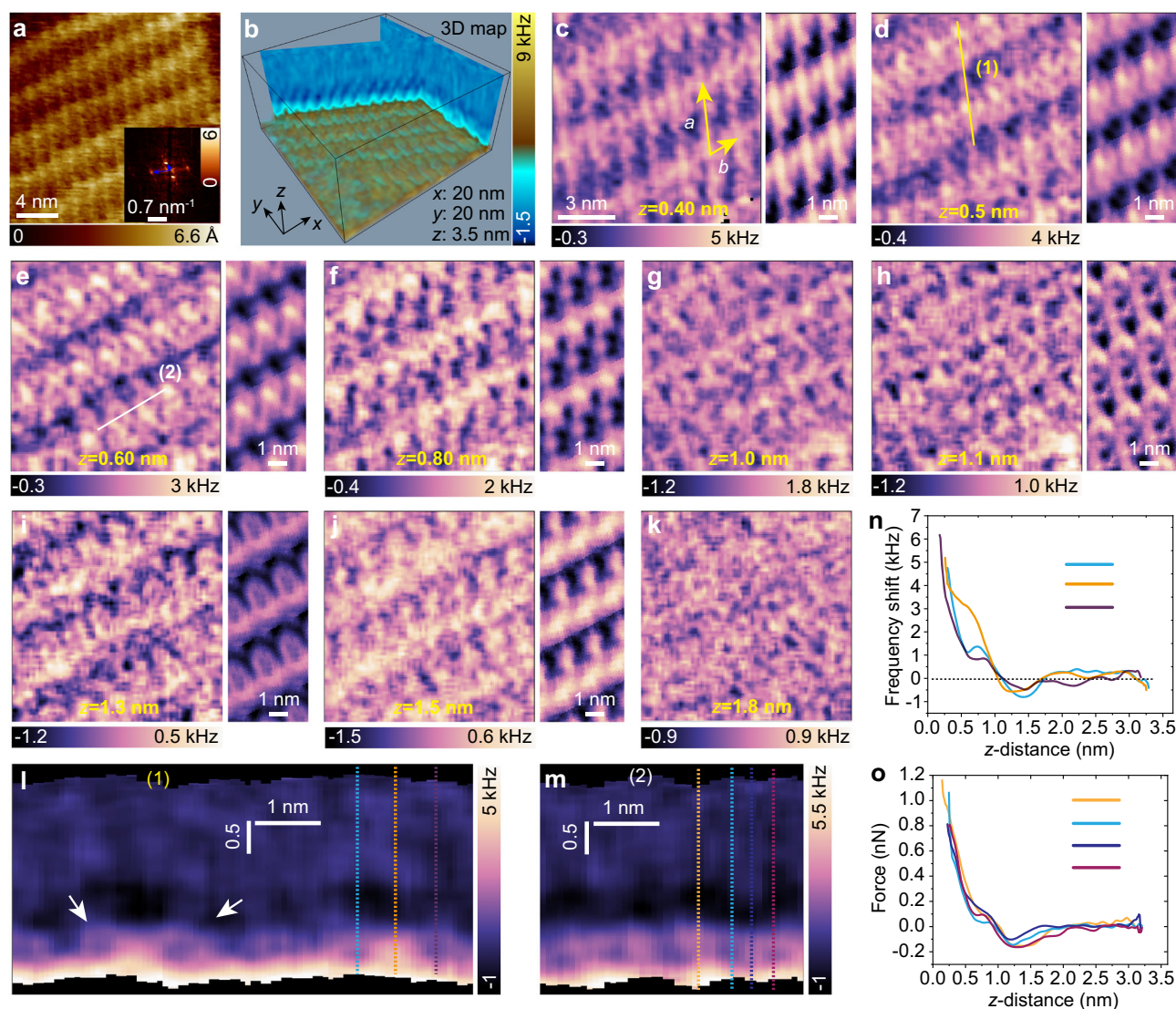


Fig. 4 | 3D map of peptide-water interface. **a** High-resolution 2D FM-AFM image showing self-assembled GrBP5-WT peptides on HOPG, observed in water. Inset: Corresponding FFT pattern highlighting the reciprocal-space periodicity of the peptide assembly. The color scale indicates the relative FFT intensity in arbitrary units. **b** 3D map of the peptide-water interface, obtained using a hydrophilic tip, showing xy and xz cross-sections of a well-ordered hydration layer above the peptide molecular lattice in panel **a**. **c–k** 2D lateral xy slices at successive heights reveal in-plane structuring of water in the hydration shell above the peptide assembly. The scale bar in panel **c** applies to all corresponding 2D xy images in panels **c–k**. The corresponding correlation-averaged 2D xy maps for each z slice are

shown in the adjacent panels with individual scale bars. **l, m** 2D vertical xz slices obtained along yellow (1) and white (2) solid lines (shown in **d** and **e**) show details of the hydration substructures across and along the organized peptide rows. An asymmetric “elephant’s head-shaped” hydration motif (white arrows in panel **l**) is observed, corresponding to the chemically distinct peptide domains (Fig. 1d). **n** Individual Δf - z curves obtained along the cyan, orange, and magenta lines in panel **l**, showing the asymmetric water structuring along the molecular axis of peptide within the assembled architecture. **o** Force-distance curves extracted using the Sader method, acquired along the dashed lines in panel **m**. Source data are provided as a Source Data file.

the xy plane progressively attenuate with increasing distance from the interface, gradually evolving toward bulk-water-like organization; and (3) These features reflect not only the lattice organization of individual peptides but also subtle sub-nm patterns arising from water interactions with the peptide’s three distinct domains—hydrophilic, hydrophobic, and aromatic (Fig. 1d). The close structural relationship between the peptide molecular organization and their interfacial hydration structures suggests that precisely engineered nanoscale architectures can encode and stabilize distinct hydration patterns, analogous to the highly organized and spatially correlated hydration networks characteristic of folded proteins.

To better visualize the molecular organization of water at the peptide–water interface, we generated force modulation (Δf) maps in 2D, both parallel and perpendicular to the surface (Fig. 4a, b). Near the

peptide surface, water molecules form specific sub-nm patterns aligned with the peptide lattice, exhibiting alternating contrast that gradually diminishes with height and fades into bulk water (Fig. 4c–k). These contrast features indicate well-ordered hydration layers extending approximately three to four water molecular diameters from the surface. The innermost layer comprises tightly bound water interacting directly with polar and charged residues, while subsequent layers represent secondary and higher-order shells characterized by progressively weaker interactions. Analysis of the lateral xy slices and corresponding FFT patterns reveals that peptide-templated planar ordering persists over an extended interfacial region while exhibiting pronounced spatial modulation with increasing distance (Supplementary Fig. 9; Supplementary Note 2). Rather than decaying monotonically, FFT spot intensities vary at different heights with certain

features weakening, disappearing, and reappearing, reflecting non-uniform hydration ordering. Within the intermediate region, the partial attenuation of selected FFT features together with the emergence of additional secondary spots suggests a progressive transition from strongly surface-directed hydration toward increasingly solvent-governed organization. Quantitative analyses of representative Δf - z profiles indicate that the interfacial water column extends 1.35 ± 0.07 nm from the nearest surface, corresponding to roughly 3–4 hydration layers (Supplementary Fig. 10). Although thermal fluctuations complicate precise delineation, transient rearrangements within the H-bond network suggest that the hydration shell may extend beyond this range. Collectively, these results reveal that, rather than forming a uniform shell around the peptide assemblies, hydration water organizes into spatially heterogeneous motifs that encode local chemical identity and persist across multiple hydration layers before gradually transitioning toward bulk-like behavior with increasing distance from the surface.

These experimentally observed hydration features are further supported by analysis of the molecular dynamics (MD) trajectories, which reveals that peptide–water hydrogen bonding occurs predominantly at negatively charged and polar residues, highlighting their central role in establishing and stabilizing the interfacial hydration network (Supplementary Fig. 11). Bond-length distributions further reveal a substantial population of short H-bonds (2.4 – 2.8 Å) at peptide–water interface, markedly contracted relative to the bulk water (Supplementary Fig. 11b). This shortening possibly reflects stronger, more directional interactions arising from confinement and templating by the peptide assembly. These contracted interactions locally stabilize the hydration structure and contribute to enhanced ordering within the first few interfacial water layers (Supplementary Note 3). Correlation-averaging analysis further confirms that the observed hydration patterns are intrinsic interfacial features. The preservation of lateral ordering motifs and hydration registry following averaging demonstrates that the dominant hydration structures arise from the underlying interfacial molecular organization rather than from local fluctuations or imaging artifacts (Fig. 4; Supplementary Fig. 12; Supplementary Note 4).

In further interrogating interfacial hydration, we captured 2D vertical maps along and across the self-assembled peptides (Fig. 4l, m). The longitudinal 2D map, aligned with the peptide orientation, reveals contrast variations spanning ≈ 3.2 nm—the length of a folded peptide—likely arising from specific interactions of water molecules with distinct chemical domains. A notable feature is an asymmetric hydration substructure resembling an “elephant’s head,” composed of localized domains arranged along the length of each peptide (Fig. 4l). The domain-specific hydration substructure is further corroborated by the Δf - z and force- z measurements, which show variations in hydration forces and interaction ranges reflecting the chemically heterogeneous peptide surface (Fig. 4n, o). The findings here align with previous 3D-AFM studies on polymeric interfaces⁶⁶, where variations in the hydration forces and the resultant contrast were attributed to the local chemical heterogeneity of surface functional groups. Thus, the hydrophobic, hydrophilic, and aromatic domains of GrBP5-WT peptide can be distinguished through their unique hydration signatures throughout the water column above the peptide. Similarly, a 2D map across multiple peptide rows (Fig. 4m; Supplementary Figs. 9, 10, 13) reveals 1.6-nm repeating contrast patterns consistent with the dimension of the peptide lattice. Here, the asymmetric hydration features likely arise from interactions between water molecules and the polar or charged side chains flanking the either side of the peptide backbone. Such localized hydration heterogeneity underscores the critical influence of the side-chain chemistry of the peptide sequence in dynamically reshaping the interfacial water structure. We note that the distinct substructural hydration features

and their spatial extent, exceeding 1 nm, are also evident in the second self-assembled peptide layer, which exhibits a more homogeneous hydration environment. This likely reflects a more extended, open peptide conformation with reduced folding propensity (Supplementary Fig. 14; Supplementary Note 5).

Tip chemistry and buffer effects on interfacial hydration

The potential effect of the AFM tip on hydration measurements^{67,68} were also investigated, where we compared hydrophilic Silicon (Si) and hydrophobic amorphous Carbon probes. The primary difference lies in vertical resolution: hydrophobic C tips yield sharper separation of oscillatory features, providing a structurally faithful representation (Fig. 5a–m; Supplementary Figs. 15–18). Conversely, hydrophilic Si tips resolve more laterally intricate features but with broader vertical contrast, driven by stronger water–tip interactions. This divergence is further reflected in the force–distance characteristics. The Si tip exhibits an enhanced long-range attractive force regime, characterized by a negative Δf response persisting over a relatively large distance from the tip’s first contact with the structured hydration layer (Fig. 4o). In contrast, the C tip displays a significantly weaker and more spatially confined attractive regime, indicating that probe chemistry strongly modulates the onset and magnitude of these initial water-mediated forces (Fig. 5k–m). Importantly, however, the overall boundary of the structured hydration shell remains invariant; for both probes, hydration features persist ≈ 1.3 nm above the peptide surface before transitioning into bulk-like behavior. These studies demonstrate that while tip chemistry influences the local interaction potential—governing vertical resolution and contrast—it does not alter the intrinsic spatial organization of the interfacial water network. This behavior is consistent with previous studies demonstrating the effects of hydrophilic and hydrophobic confinement on hydration-force profiles⁶⁸.

The averaged 1D Δf -distance profiles along both the x - and y -axes (Fig. 5j) reveal the onset of tip–sample contact at ≈ 0.20 – 0.25 nm, followed by three pronounced oscillatory features and a weaker outer peak that progressively decays into the bulk-water regime, delineating a structured hydration shell extending ≈ 1.3 nm and above from the interface. The apparent valley-to-valley spacings extracted from the averaged profiles ($N=201$) are larger ($d_1 = 0.36 \pm 0.01$ nm, $d_2 = 0.42 \pm 0.02$ nm, $d_3 = 0.42 \pm 0.09$ nm) than the molecular diameter of water (≈ 0.30 nm), likely reflecting lateral averaging across a chemically and structurally heterogeneous peptide interface (Supplementary Fig. 17; Supplementary Note 6). These expanded apparent spacings likely arise from the superposition of distinct local hydration environments. Contributions from peptide chains with different lateral packing densities, vertical positions, and conformational states, together with overlapping hydration structures associated with chemically distinct surface moieties, broaden the averaged hydration response. A similar broadening of apparent hydration-layer spacing has been reported in 3D-AFM studies of lipid membranes, where interfacial roughness and molecular fluctuations were shown to increase the apparent separation between hydration layers⁶⁹. Beyond the first hydration layer, this effect is further amplified by a progressive broadening and attenuation of the oscillatory response, arising from nanoscale structural heterogeneity and thermal fluctuations, rather than any intrinsic change in the molecular length scale of water or contributions from ion adsorption or contamination. In contrast to HOPG, which impose laterally uniform hydration layering through rigid templating, the peptide assembly forms a dynamically fluctuating and structurally heterogeneous interface strongly coupled to the surrounding solvent, giving rise to anisotropic and progressively diffuse hydration structures beyond the first oscillation. Consistent with this interpretation, individual force–distance curves acquired at selected positions (Fig. 5l) exhibit interlayer spacings of ≈ 0.30 – 0.34 nm, consistent with the established molecular dimensions of water. To

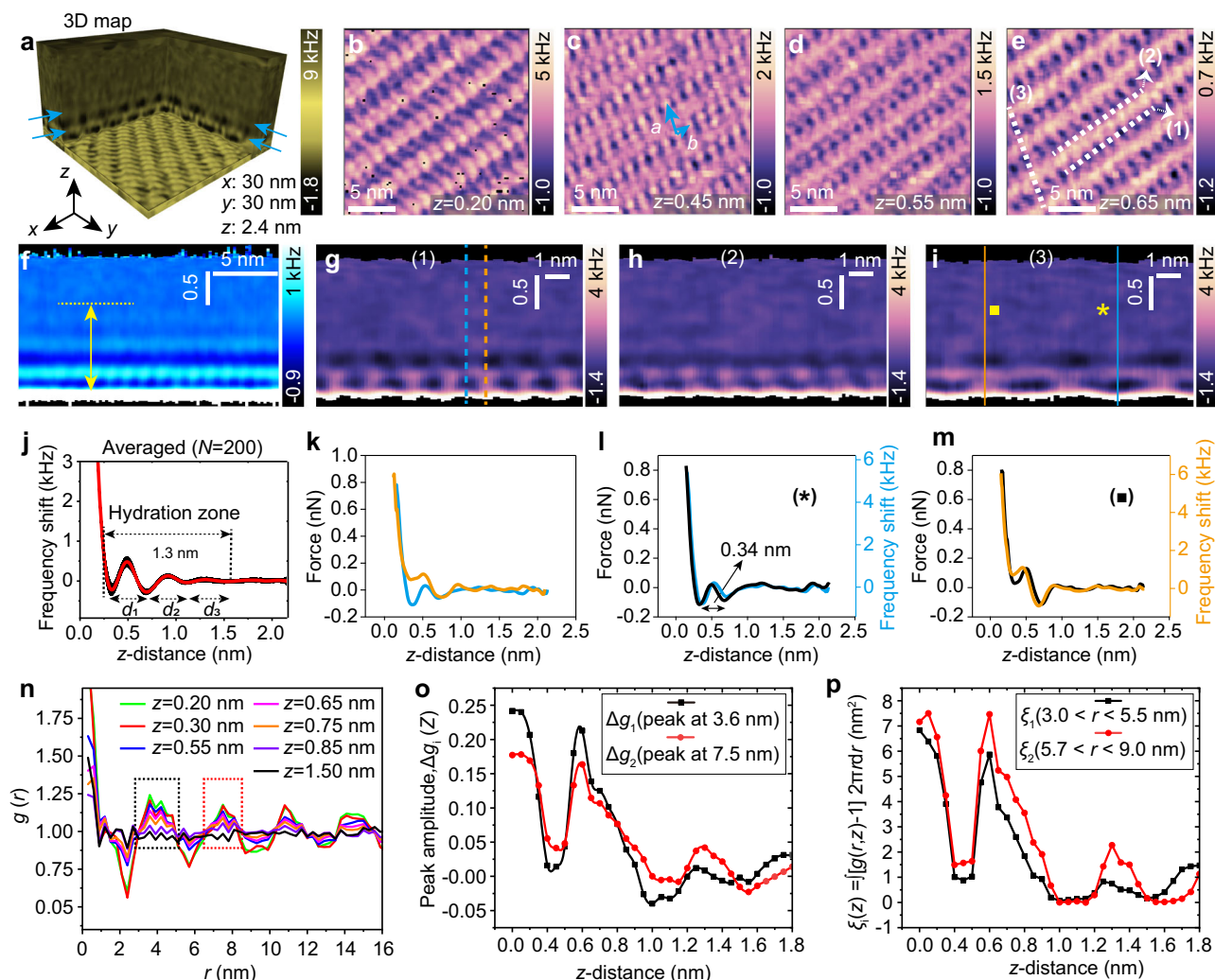


Fig. 5 | Sub-molecular mapping of peptide hydration using a hydrophobic carbon tip. **a** 3D-AFM image of the peptide-water interface, obtained using a hydrophobic carbon tip. The overlaying 2D *xy* and *xz* cross-sections illustrate a well-ordered hydration layer covering the entire self-assembled peptide domain. **b–e** Lateral *xy* slices at different heights from the peptide surface show in-plane structuring of water in the hydration shell. **f** The averaged 2D vertical map. **g–i** The 2D maps, presented as vertical *xz* slices along lines 1–3 (shown in panel **e**), highlight the detailed multilayered hydration substructures along (lines 1–2) and across (line 3) the peptide rows. **j** The individual Δf -*z* curves (black) and their average curve (red) show several maxima, corresponding three to four hydration layers.

k–m Force curves, derived using the Sader method along dashed lines (colored lines in panel **g** were used in panel **k**, and lines in panel **i** were used for panels **l** and **m**, marked with an asterisk and square symbol, respectively). **n** Representative in-plane pair distribution functions, $g(r)$, for individual hydration layers, with the two selected peak regions highlighted. The amplitudes ($\Delta g_i(z) = g_i^{\text{peak}}(z) - 1$) of two representative $g(r)$ peaks at ≈ 3.6 and 7.5 nm are quantified in panel **o** as a function of distance from the surface. **p** The second metric is the integrated excess correlation $\xi_i(z) = \int [g(r,z) - 1] dr$, evaluated over the *i*-th peak window and restricted to regions where $g(r,z) > 1$, corresponding to the area under each peak above the bulk reference. Source data are provided as a Source Data file.

quantitatively validate the short-range origin of these oscillations and rule out long-range electrostatic or van der Waals artifacts, we analyzed the force decay characteristics. Fitting individual force curves to an exponential decay, $F(d) = F_0 e^{-d/\lambda}$, yields a highly localized characteristic decay length of $\lambda \approx 0.06$ nm, with the force decaying completely within ≈ 0.15 nm (Supplementary Fig. 19; Supplementary Note 7). This localized response is characteristic of short-range, non-DLVO hydration forces^{70,71}, confirming that the oscillatory features reflect genuine interfacial solvent restructuring.

The cross-sectional *xz* and *yz* maps reveal conformal layered substructures that are commensurate with the surface topography of the assembled peptides (Fig. 5f–i), indicating a high degree of structural registry between the peptide assembly and their hydration structure. Beyond the immediate interface, the hydration structure becomes progressively more diffuse, with increasingly loosely bound water molecules forming extended hydration domains that gradually converge toward bulk-water behavior. Molecular ordering within the

hydration shells and its distance-dependent attenuation were quantitatively assessed using pair distribution function, $g(r)$, and first-order FFT spot intensity analyses (Fig. 5n–p; Supplementary Figs. 20–22; Supplementary Note 8). For each *xy* slice extracted from the 3D-AFM measurements, the two complementary metrics, the peak amplitude, $\Delta g_i(z)$, and peak-integrated excess correlation, $\xi_i(z)$, were used to quantify local and cumulative spatial correlations, respectively. Together, these metrics provide a quantitative framework for characterizing the progressive evolution of hydration-layer organization from the peptide interface toward the bulk solvent. For both the first and second correlation peaks, $\Delta g_i(z)$ and $\xi_i(z)$, exhibit closely similar distance-dependent behavior (Fig. 5o,p). At short distances ($z \approx 0.3$ nm), both metrics show high values, reflecting strong interfacial confinement and enhanced spatial correlations among hydration water molecules. With increasing distance, both metrics decrease and reach a minimum at $z \approx 0.4$ – 0.5 nm, indicating an interlayer transition region with partially disrupted structural coherence. Their partial recovery near $z \approx 0.6$ nm

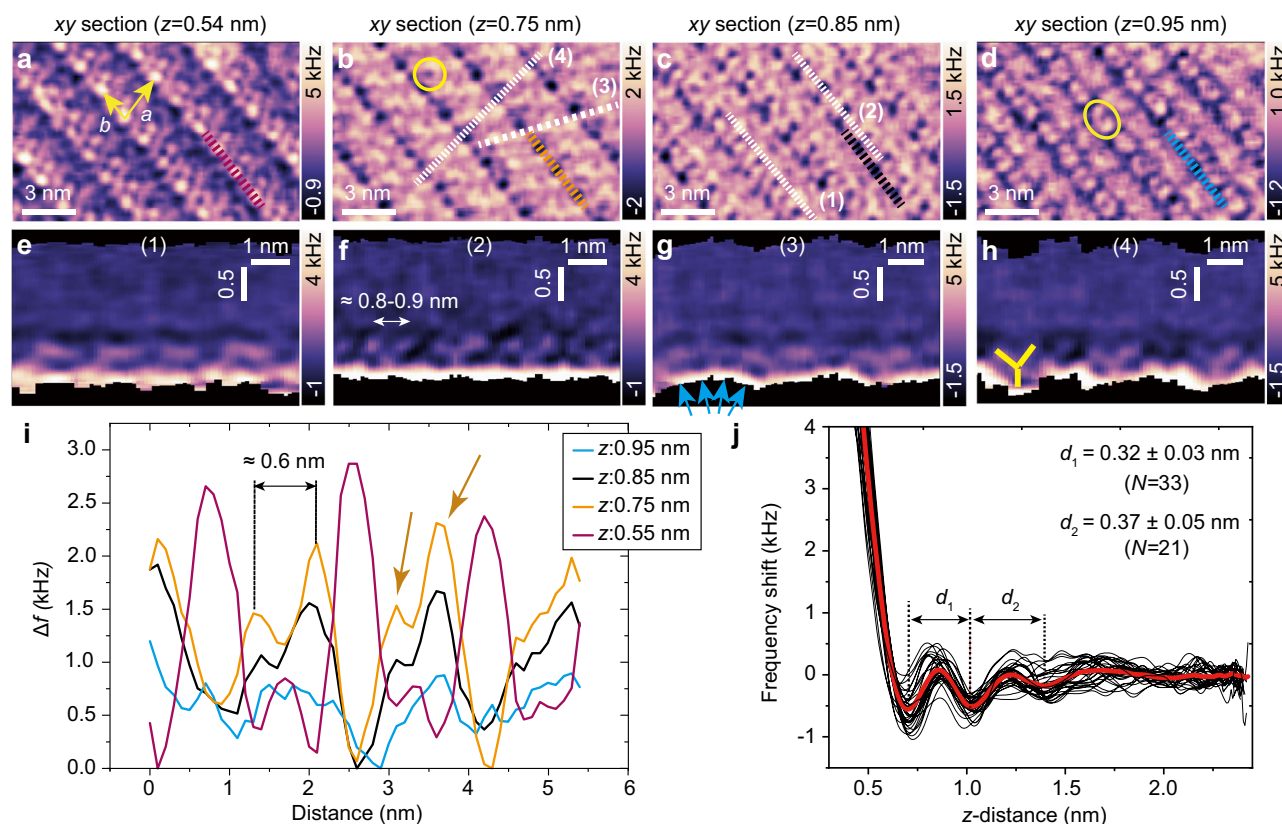


Fig. 6 | 3D hydration structure of the peptide assembly in PBS buffer. a–d The 2D *xy* slices at different heights from the peptide surface show in-plane structuring of water in the hydration shell (acquired in PBS buffer). **e–h** Vertical *xz* slices along lines (1)–(4) reveal sub-structural details in the hydration shell along and across the peptide rows. The cyan arrows in panel **g** denote the distinct substructural hydration features localized along the individual peptide within the assembly. **i** Δf line profiles along colored dashed lines in the *xy* slices in **a–d**. Splitting of the Δf

profile at a relatively higher height is observed with a separation of approximately 0.6 nm, indicating interactions between interfacial water molecules and the peptide side chains. **j** Individual Δf -*z* profiles (black) and the averaged profile (red), revealing characteristic hydration layer spacing of $d_1 = 0.32 \pm 0.03$ nm ($N=33$) and $d_2 = 0.37 \pm 0.05$ nm ($N=21$) (mean $\pm$ s.d.). Note that a more complete set of slices is presented in Supplementary Fig. 23, from which the representative images shown above were sampled. Source data are provided as a Source Data file.

suggests non-monotonic decay of the hydration structure, accompanied by local reorganization between neighboring layers. At larger distances, both Δg and ξ progressively decrease; however, a weak but statistically detectable third diffuse hydration shell remains evident near $z \approx 1.25$ – 1.30 nm, revealing the persistence of interfacial structural correlations across multiple hydration layers before their eventual decay toward bulk-water behavior. Beyond this distance, the profile exhibits only minor fluctuations, consistent with a progressive transition toward bulk-like solvent behavior. The non-monotonic decay of interfacial water ordering is further supported by FFT peak intensity analysis, which closely tracks the evolution of the corresponding $g(r)$ peak height and confirms the agreement between reciprocal-space and real-space measures of lateral ordering within the hydration layers (Supplementary Fig. 22d–f). Overall, comparison of hydrophilic and hydrophobic probes demonstrates that, although tip chemistry influences imaging contrast and force-response characteristics, the observed hydration structures appear to be intrinsic properties of the peptide–water interface rather than artifacts arising from tip–water interactions.

To examine how dissolved ions influence interfacial hydration and interact with peptide side chains, we further analyzed the peptide hydration structure in PBS buffer (Fig. 6a–d; Supplementary Figs. 23–25; Supplementary Note 9). The hydration landscape exhibits an asymmetric Y-shaped pattern aligned with the orientation of the peptide side chains (Fig. 6f, h), more clearly resolving the sequence-specific chemical domains along the peptide. The 2D vertical hydration map along the peptide axis reveals three distinct substructural

domains aligned along the peptide length on the surface (Fig. 6g). Consistent with this spatial heterogeneity, circular and ellipsoidal hydration features emerge at larger distances from the peptide surface (Fig. 6a–d), in contrast to the more localized motifs observed at the peptide–water interface (Figs. 2 and 4). The distal features likely arise from structured water rather than the peptide’s surface topography alone. The Δf line profiles across the peptide width show asymmetric splitting at the intermediate and larger distances. This splitting is more pronounced in the orange and black traces, with the peak separations of ≈ 0.6 nm matching the peptide width (Fig. 6i). The distinct, asymmetric, contrast on each side of the peptide could be indicative of the localized, but different, water structuring around the chemically distinct side chains (Fig. 6f). In short, the observed hydration patterns suggest that water does not form a uniform shell around the peptide but, instead, organizes locally, forming nuanced substructure, in response to distinct chemical domains on the peptide surface. Strong interactions between Na^+/K^+ ions and the acidic residues aspartate (D) and glutamate (E) appear to reorganize the solvation structure above the peptide, whereas the self-assembled peptide pattern remains largely intact due to weaker ion interactions with tyrosine (Y) and serine (S) residues in the peptide-binding domain. The first hydration layer spacing of 0.32 ± 0.03 nm is consistent with the characteristic spacing of structured interfacial water reported in dilute aqueous environments, supporting its assignment to the primary hydration shell directly associated with the peptide backbone at the surface (Fig. 6j). In contrast, the broader spacing of 0.37 ± 0.05 nm likely reflects a more heterogeneous hydration environment arising from overlapping

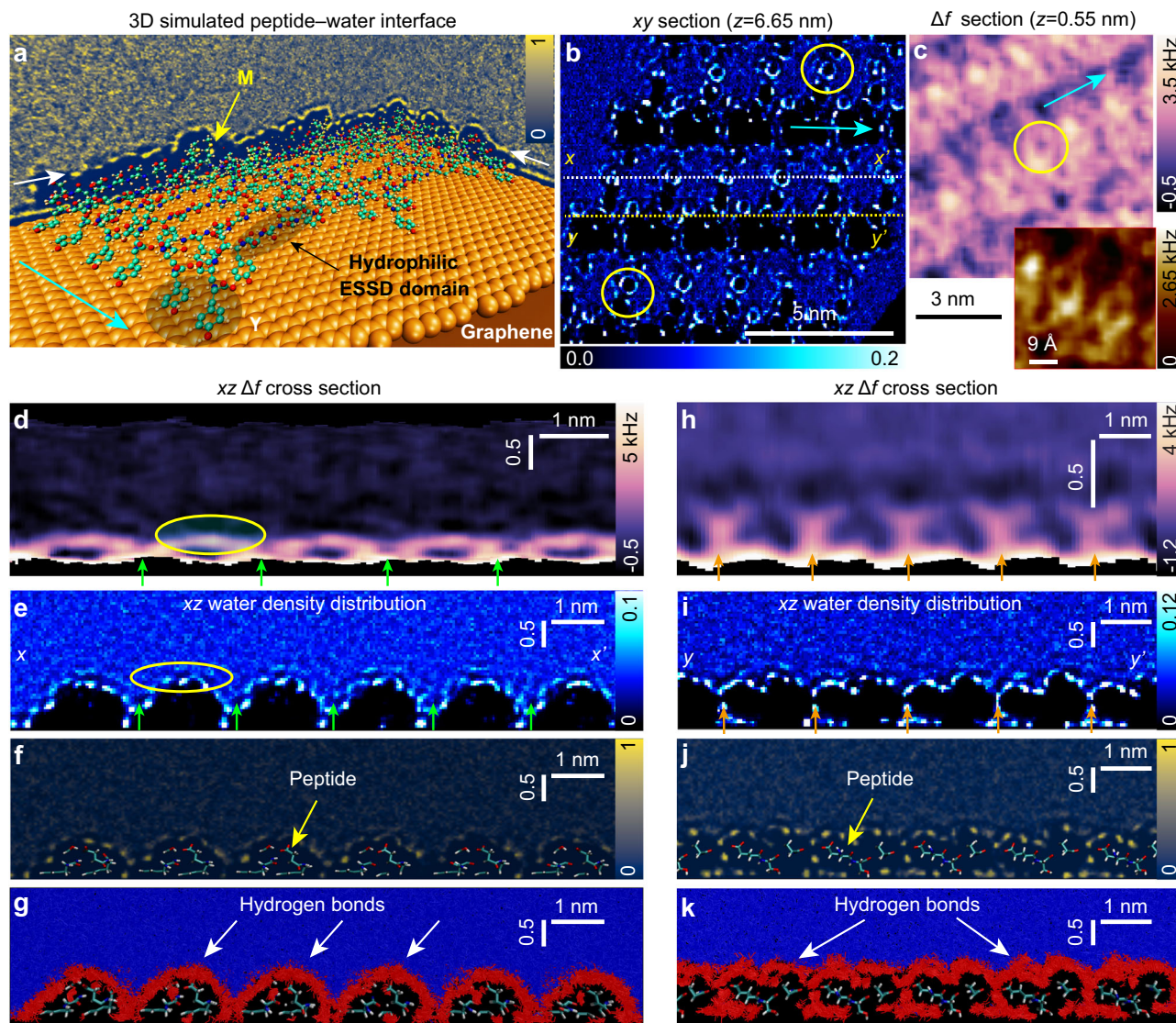


Fig. 7 | Molecular dynamics analysis of water density distribution and hydration motifs at the peptide interface. **a** Representative 3D visualization of the simulated peptide–water interfacial model system, shown together with xz and yz views overlaid on the underlying peptide assembly on the graphene lattice. **b** Simulated 2D xy slice showing the localized in-plane water O-density distribution over the peptide assembly at $z = 6.65$ nm (see Supplementary Fig. 28a). **c** Experimental 2D xy Δf slice extracted from the 3D map (Fig. 4b, $z = 0.55$ nm), showing circular molecular hydration features at sub-nanometer resolution, similar to those observed in the simulated image in **b**. **d, h** 2D vertical xz Δf slice taken along the peptide rows at two different lateral positions. **e, i** 2D vertical density profile of water O-atoms above the peptide nanostructures, extracted along the x - x' and y - y'

directions (white and yellow dashed lines) indicated in panel **b**, respectively. **f, j** Representative visualization generated from 100 MD snapshots, showing the spatial distribution of water around the peptide self-assembled structures. The color scale in panels **f** and **j** represents the normalized water oxygen density. The underlying peptide molecular assembly is indicated by yellow arrows in panels **f** and **j**. **g, k** Corresponding H-bonds formed between the peptide and surrounding water molecules shown in panels **f** and **j**, respectively. In panels **g** and **k**, color coding represents distinct water populations within the hydration layer: red denotes water molecules participating in hydrogen-bond networks at the peptide–water interface, while blue denotes other water molecules in the surrounding solvent region.

hydration structures above the residues with different degrees of surface exposure and vertical positions, together with broadening induced by thermal and conformational fluctuations of the side chains, similar to that observed in pure water.

Molecular dynamics analysis of interfacial water

To establish a molecular basis for understanding the experimental results, we performed MD simulations using the structure identified by the NNP structure search, which was replicated into a 6×5 supercell and solvated with water (Methods). The simulated 3D structural map along with 2D slices (Fig. 7a, b) shows the spatial distribution of water O-atoms near the peptide surface (Supplementary Figs. 26–28). Close to the interface, water molecules adopt highly ordered spatial

distributions that follow the folded peptide topography and local side-chain organization (Fig. 7a, b; Supplementary Figs. 26–31; Supplementary Note 10). The resulting high-density regions reproduce the circular and ellipsoidal hydration features observed experimentally, establishing direct correspondence between the AFM measurements and the simulated water-density distributions (Fig. 7b, c). Extending this correspondence, the two recurring hydration architectures observed experimentally—an arch-shaped substructure containing central voids and periodically arranged Y-shaped hydration motifs (Fig. 7d, h)—are likewise reproduced by the MD-derived water-density maps and representative hydration snapshots. These features primarily arise from interactions with the polar side chains within the hydrophilic domain of the folded peptide (Fig. 7d–k). Importantly, the

vertical hydration motifs across different orientations are consistently captured in the MD simulations, supporting the experimental observations (Supplementary Figs. 29, 30). Beyond local water–peptide interactions, the vertical hydration organization is governed by the 3D architecture of the peptide crystal on graphite, the orientation of surface-exposed side chains, and the hydrogen-bonding network formed with surrounding water molecules (Fig. 7g, k). Near the surface, strongly packed water molecules align with the peptide orientation, decorating the backbone and side-chain residues and forming water bridges between the topographically protruded domains encompassing the IMVT domain near the C–N terminal junction (Supplementary Figs. 26–31; Supplementary Note 11). With increasing distance from the interface, the circular hydration features emerge before the peptide-specific water organization gradually diminishes. Further away from the surface, the hydration structure transitions to a pattern aligned with the longitudinal axis of the peptide nanowire (cyan arrow, Fig. 7a), with density preferentially concentrated at the C–N junction. This preferential localization is consistent with the elevated topography and increased solvent exposure of the methionine (M) residue within the IMVT domain, which is more accessible to the surrounding solution, whereas the central hydrophilic regions remain in closer proximity to the surface. Consequently, the sequence-specific water interactions extend further along the junction. Additionally, the localized water structuring near the hydrophobic domains may arise from two parallel peptides with slightly offset orientations, creating interfacial cavities that facilitate hydration. A reduction in water density near the side chain (M) further highlights domain-specific hydration behavior (Supplementary Figs. 27 and 28). It should be noted that, although the first hydration layer is clearly resolved in Fig. 7, the second-layer density modulation remains relatively weak. This primarily reflects limitations of the TIP3P water model, which underestimates structural ordering beyond the first hydration shell. Simulations using the OPC water model⁷², which more accurately reproduces water structure beyond the first hydration layer, recover a more pronounced second hydration layer while preserving the overall hydration architecture (Supplementary Figs. 32–39). Consistently, explicit-tip free-energy simulations reveal a more extended structured hydration region, in closer agreement with the AFM measurements (Supplementary Figs. 34–38). The multilayer nature of the hydration structure is further supported by tetrahedral order-parameter analysis, which shows recovery of bulk-like water structure only at distances of $\approx 8\text{--}9\text{ \AA}$ from the peptide surface (Supplementary Fig. 32; Supplementary Note 12). Remaining differences likely arise from the convolution of tip and sample hydration structures in AFM, as well as limitations in force-field accuracy and limited sampling of peptide conformational fluctuations. Taken together, these results support the conclusion that peptide-induced hydration ordering extends across multiple interfacial water layers.

Discussions

The work here establishes a sub-nm level spatially resolved, force-mapped three-dimensional architecture consisting of a structurally graded hydration shell, that is commensurate with the structural features of a self-organized peptide molecular crystal assembled on a solid surface. AFM-based imaging of hydration shell has previously been applied to biomolecular systems such as DNA⁷³. These studies showed the feasibility of visualizing biomolecular hydration using 3D-AFM. The ordered peptide crystal investigated here extends this capability by enabling the resolution of residue-specific hydration architectures and their evolution across multiple hydration layers. Combined with quantitative analyses and molecular simulations, the 3D imaging approach provided insights into how local biomolecular chemistry shapes the spatial organization of interfacial water and establishes a framework for investigating chemically encoded hydration landscapes at biomolecular interfaces.

The observed molecular patterns in the hydration substructure are most pronounced near the peptide surface that progressively diffuse with increasing distance into the bulk solution. This behavior of the hydration substructure reflects the weakening organization of the overall H-bonded molecular network probably due to the gradual loss of long-range electrostatic and directional H-bonding among the water molecules and peptides' side chains. These features represent time-averaged contrast variations arising from rapidly fluctuating water configurations transiently organized by interfacial confinement and surface templating. Accordingly, the apparent structural regularity reflects enhanced spatial correlations within a dynamic hydrogen-bond network governed by peptide sequence and molecular organization with possible formation of locally ordered distinct structured water phases. This interpretation is consistent with spectroscopic, scattering, and computational studies showing that, although hydration water generally remains dynamically active near the surface¹⁵, it exhibits significantly slowed dynamics relative to the bulk water.

Vertical and horizontal slices taken across the hydration shell further reveal peptide-specific hydration architectures shaped by discrete chemical domains displayed on the peptides. Near the surface, water forms highly structured and possibly rigid, multilayer molecular organization that progressively relaxes into more disordered, spatially constrained networks with increasing distance from the peptide/water interface. This organization extends to 1.3 nm, and beyond, from the peptide surface and retains peptide-specific structural signatures arising from dipolar, electrostatic, and hydrogen-bonding interactions with the water molecules. Furthermore, the hydration landscape exhibits increased lateral heterogeneity, generating chemically specific, site-dependent hydration fingerprints that extend beyond the conventional averaged descriptions of interfacial water and may encode functional information associated with the underlying amino acid sequence of the peptide. The spatial heterogeneity and chemical specificity of these hydration architectures could establish the interfacial water as an active determinant in the biomolecular recognition processes. By encoding sequence-dependent hydration fingerprints, water can locally modulate free-energy landscapes and, therefore, intermolecular interactions, that would provide an additional layer of molecular information beyond the direct residue–residue contacts between a ligand and its receptor. These observations suggest that hydration structure itself could contribute to the functional encoding of protein interfaces, linking amino acid sequence, surface chemistry, and molecular recognition through chemically, physically, specific water organization within the hydration shell.

The simplified approach here provides a physically (material components) and chemically (interactions) controlled platform for isolating how specific amino-acid functionalities could shape local hydration structures, although the ordered peptide assemblies studied differ substantially from the dynamic, crowded, and structurally heterogeneous environments found in living systems. By immobilizing the peptides that commensurately self-assemble on the relevant solid substrate (here, cleaved graphite) and, thereby, suppressing large-scale conformational fluctuations, the present system enables direct, sub- $\text{\AA}$ spatial resolution of residue-specific hydration landscapes that are typically obscured in ensemble-averaged 3D biological measurements. In living systems, hydration patterns are continuously modulated by thermal motion, conformational rearrangements, and molecular crowding, which rapidly diminish long-range spatial coherence. Nevertheless, extensive experimental and computational studies have shown that local hydration motifs remain strongly governed by residue-level chemistry, including hydrophobicity, polarity, charge, and hydrogen-bonding capability^{74,75}. Consistent with this view, recent Raman microspectroscopy studies identified a small population of

structurally distinct biointerfacial water in living cells⁷⁶, corresponding to approximately one hydration layer surrounding soluble proteins. The hydration features identified here should, therefore, be interpreted not as biologically persistent long-range water ordering, but rather as local chemically encoded hydration signatures that may persist within the - spatially and temporally - fluctuating biomolecular environments. Beyond their mechanistic relevance to molecular recognition and water-mediated interactions, the high-resolution hydration maps of peptides presented here also provide experimentally constrained benchmarks for validating molecular simulations of interfacial water structure and dynamics.

While our findings offer direct visualization of hydration structures at molecular resolution, several critical questions remain. The dynamic coupling between hydration shell fluctuations and protein conformational changes—long proposed as a driver of biological function—still lacks sufficient spatiotemporal resolution. Although we show here how the peptide sequence and side-chain chemistry encode specific hydration motifs, the extent to which these motifs mediate long-range communication, cooperative binding, and ligand-receptor interaction, still needs to be studied^{12,15,77,78}. Most current models largely disregard the heterogeneity of hydration behavior in crowded or confined environments, such as membranes and intracellular compartments^{12,17,18}. Moreover, predictive algorithms for protein structure and function rarely incorporate explicit hydration sub-architectures, despite accumulating evidence that structured water plays a decisive role in molecular recognition^{12,15,42,43}. Although our findings highlight the potential role of the water substructures in the hydration shells in shaping protein folding, function, and recognition^{34,79}, addressing the persistent knowledge gaps in their spatial and temporal characterization remains essential for advancing predictive protein design, decoding cellular mechanisms, and deepening our fundamental understanding of biomolecular behavior.

Our results support a view in which hydration contributes directly to the structural organization of proteins, forming sequence-dependent interfacial architectures that are closely associated with the underlying molecular framework. This integrated view of proteins as superstructures, including protein itself and its specifically organized hydration structure, provides a framework for understanding molecular recognition, enzymatic activity, and drug interactions. The ability to modulate interfacial hydration through surface chemistry offers a design principle for engineering bio-interfaces and biomimetic materials with tunable interaction, catalytic, and antifouling properties. Future work should focus on extending these high-resolution hydration maps to dynamic biological environments, exploring how hydration-based protein superstructures evolve under mutations, conformational fluctuations, crowding, and ligand binding. Such studies will be essential for incorporating hydration into predictive models of protein functions and for harnessing hydration-driven design principles in protein engineering.

Methods

Peptide solution preparation

20 mg of GrBP5-WT peptide powder (with purities >95%) was provided by GL Biochem (Shanghai) Ltd. The amino acid sequence of the GrBP5 peptide is NH₂-IMVTESSDYSSY-COOH, and its molecular weight was determined to be 1381.47 g mol⁻¹ through mass spectrometry and high-performance liquid chromatography analyses. To prepare peptide solutions with concentrations of 250 μM, the peptide powder was dissolved in either phosphate buffer or ultrapure water. These prepared peptide solutions were then stored in a -20 °C refrigerator. To achieve the desired GrBP5-WT concentrations, the stock solution was further diluted in ultrapure water to yield concentrations of 150, 100, 20, 10, 5, and 1 μM, respectively. Milli-Q deionized water with a resistivity of 18.2 MΩ cm was used throughout the experiments.

Sample preparation for AFM observation

A freshly cleaved HOPG crystal with a mosaic spread of 0.4°±0.1° (purchased from MikroMasch), obtained through mechanical exfoliation using Scotch tape, was used as the substrate for peptide assembly in either water or PBS. The stock peptide solutions were warmed to room temperature, and then 120 μL of each peptide solution was directly deposited onto the HOPG surfaces. The solutions were left to adsorb for 5–10 min at room temperature. The molecular structures of the self-assembled peptide nanostructures were assessed using a custom-built FM-AFM system in a liquid environment.

FM-AFM experiments

The high-resolution molecularly resolved AFM images of peptide assemblies on the HOPG were obtained using a custom-built frequency modulation AFM (FM-AFM) system operating in liquid environments, and equipped with an ultralow-noise cantilever deflection sensor⁸⁰. The oscillation of the cantilever was driven by photothermal excitation at resonance with the amplitudes set at <0.25 nm. The AFM scanning process was controlled using a commercial SPM controller (ARC2, Asylum Research). A constant cantilever oscillation amplitude was maintained by adjusting the excitation signal amplitude using a commercially available controller (OC4, SPECS). The AFM was operated in the constant frequency shift (Δf) mode, where the tip-sample distance was adjusted such that Δf was kept constant. AFM image data were collected using a commercially available silicon cantilever (160AC-NG, OPUS) with a nominal spring constant of 26 N m⁻¹ and a nominal apex radius of ≈7 nm. We obtained cantilever eigen frequencies in the range of 125–142 kHz, and quality factors of 7–8 in pure water. In most cases, the cantilevers were coated with Si (15 nm) by a dc sputter coater (KST-CSPS-KFI) to improve the tip-apex stability and remove the tip-apex contaminants. Additionally, we used a carbon growth cantilever to explore the influence of tip characteristics on the 3D hydration structures, i.e., effect of hydrophobic tips. The cantilever spring constants were calibrated using the thermal noise method after each experiment. AFM data were processed and analyzed using the WSxM image analysis software. The raw AFM data were post-processed by flattening and plane-fitting to eliminate background tilt where necessary.

3D-AFM characterization of interface structures

We performed 3D-AFM force mapping experiments to characterize the 3D local hydration structures around the self-assembled peptide structures on HOPG in water and PBS buffer. The 3D Δf maps of the peptide-water interfaces were obtained using the 3D force mapping technique, where a fast sinusoidal signal was additionally applied to the z-piezo to control the tip's z position during image acquisition and force mapping. The applied z-piezo signal was synchronized with the lateral xy scans of the tip, where Δf was recorded for each tip position, while the average tip-sample distance was regulated so that the average value of the Δf set-point was kept constant. With this way, one can obtain a map in which each pixel in the 3D interfacial space corresponds to a Δf . As the tip approached the surface, an oscillatory force acts on the tip due to the displacement of the structured water layer at the interface. A 3D- Δf map covering an area of (20–30) × (20–30) nm² was acquired by real-time recording of Δf with respect to the tip positions in the 3D interfacial space at the interface between the self-assembled peptide nanostructures and water. The typical resolution of the 3D Δf datasets was 128 × 128 pixels laterally and 256 pixels vertically, prior to data interpolation, as we applied in some cases. The cantilever oscillation amplitudes were set in the range of 0.1 to 0.2 nm, which is smaller than the size of a water molecule, enabling us to resolve the distribution of local hydration layers. The frequency and amplitude range of the z modulation signal during the 3D-AFM force mapping were: 195.3 Hz and 2–3 nm, respectively.

3D-AFM data processing

We used a homemade data analysis software built on the LabVIEW platform to process the large-size 3D force mapping raw data, allowing extraction of 2D section maps at various vertical and lateral positions, 3D visualization of force mapping data, and extraction of single force curves. The following steps were executed to obtain the 3D- Δf data maps. The baseline correction was initially performed for a region far from the surface, where force values at far tip-sample distances were aligned to zero. The slope of the surface was then corrected by subtracting the average height from each line scan. Then, the height correction was performed. Finally, the 3D Δf data were filtered using a 3×3 averaging filter. When necessary, we increased the resolution of the 3D- Δf data through linear interpolation.

Preparation of carbon tips

An amorphous carbon tip was grown on the apex of the original AFM tip by electron beam deposition (EBD) using a scanning electron microscope (SEM) (Supra 40VP, Zeiss, Oberkochen, Germany). After deposition, the apex of the newly fabricated tip was further sharpened by Argon plasma etching using a plasma cleaner (Tergeo, PIC Scientific).

Determination of the pair distribution function, $g(r)$

2D frequency-shift (Δf) slices were first normalized using min-max scaling:

$$\Delta f' = \frac{\Delta f - \min(\Delta f)}{\max(\Delta f) - \min(\Delta f)}, \quad (1)$$

The normalized images ($\Delta f'$, ranging from 0 to 1) were then transformed using a sigmoid weighting function:

$$w = \frac{1}{2} \left\{ \tanh \left(\frac{\Delta f' - \Delta f'_{\text{threshold}}}{s} \right) + 1 \right\}, \quad (2)$$

This transformation preserves the overall image contrast while enhancing the discrimination of hydration features. Optimal performance was obtained for $\Delta f'_{\text{threshold}} = 0.6$ and $s = 0.15$, where $\Delta f'_{\text{threshold}}$ and s denote the threshold and smoothing parameters, respectively. The pair distribution function, $g(r)$, was then calculated from the weighted images according to the method described in ref.⁸¹:

$$g(r) = \frac{\eta(r)X^4}{\eta' \delta r \{ \pi X^2 r - 4Xr^2 + r^3 \}}, \quad (3)$$

where X is the lateral dimension of the analyzed image, δr is the radial bin width, which was set to 0.3 nm, $\eta(r)$ denotes the weighted number of point pairs with separation distances within $r \pm \delta r/2$, and η' is the total weighted pair count used for normalization. Representative processed images are shown in Supplementary Fig. 20.

Computational details

Our computational approach focused on directly determining the final, experimentally observed state of the peptide assembly. To do this, we generated a large number of diverse unit cells formed by two GrBP5-WT peptides. Among these, the ones that closely matched the experimentally measured unit cell parameters were selected. Finally, a unit cell that also visually matched the observed patterns was considered for calculating the water density. By exclusively relying on the comparison with high-resolution 3D-AFM data, this approach allowed us to avoid many hurdles which would have come from a purely ab-initio search of a pathway leading to the observed self-assembled state. Similar searches have been unsuccessful, even when utilizing state-of-the-art enhanced sampling techniques⁸¹. This is likely owing to the need to access

timescales exceeding tens of μs , but possibly also to the limited accuracy of current classical molecular dynamics force fields (FFs). These FFs have been used to simulate individual proteins or peptides interacting with various carbon nanomaterials^{82–84}, but their quantitative accuracy in modeling ordered assemblies on HOPG has not been demonstrated.

Neural network potential for GrBP5-WT

As a first step to enable the generation of realistic unit cells, we trained a NequIP⁸⁵ NNP specifically for GrBP5-WT on HOPG. NNPs allow one to compute interatomic forces with an accuracy close to DFT, but for a fraction of the computational cost. To build a NNP training dataset which would faithfully represent our target atomic environments, smaller peptides built using only the amino-acids contained in GrBP5-WT were created (in groups of 2, 3 or 4), and randomly positioned on HOPG. In some of these cases, several water molecules were also added around the peptides. A total of 1156 structures were created, among which 1131 were neutral and 25 were negatively charged, since they contained aspartic or glutamic acid which were deprotonated (experiments were conducted at physiological pH). Single-point DFT calculations were carried out in VASP⁸⁶ using the optB86b-vdW functional⁸⁷, to adequately account for dispersion interactions. For the charged structures, we implemented the self-consistent potential correction method (SCPC)⁸⁸ in VASP, which provides better correction guarantees compared to the common approach of adding a jellium countercharge and using large supercells. The structures, along with their corresponding DFT energies and forces, were used to train the NNP, with 1029 configurations assigned to the training set and 127 to the validation set. The NNP was trained using NequIP (version 0.6.0). The network comprised four interaction layers with a feature multiplicity of 32, including both even- and odd-parity features up to a rotation order of $l=2$, a cutoff radius of $r=7.5 \text{ \AA}$, a radial basis of 8 trainable Bessel functions with a polynomial-cutoff exponent of $p=6$, and a two-layer radial network with 64 hidden neurons per layer and self-connections enabled. A per-species rescaling was applied, with the energy shift and force scale initialized, identically for all species, to the dataset mean per-atom energy and to the root-mean-square of the dataset force components, respectively. Optimization used a batch size of 1, an initial learning rate of 0.005, and a PerSpeciesLLoss force term (coefficient 10000.0; energy-loss coefficient 1.0), with an exponential moving average of the model weights (decay 0.99) used for validation. The learning rate was configured to halve after 100 epochs without improvement in the validation loss, and early stopping with a patience of 50 epochs was enabled. Training was carried out for 48 h on an NVIDIA A100 GPU, during which the model completed 696 epochs, yielding a validation force mean absolute error (MAE) of $48.9 \text{ meV \AA}^{-1}$ and a validation energy MAE of $0.956 \text{ meV atom}^{-1}$.

Structure search

A linear structure for GrBP5-WT was built using Avogadro⁸⁹, considering deprotonated aspartic and glutamic acids and using NH_3^+ and COO^- for, respectively, the N and C termini. From this structure, 10000 different GrBP5-WT rotamers were generated by randomly rotating all side-chains around the backbone. Finally, each of these rotamers constituted the basis to create a two-GrBP5-WT unit cell, among which 2000 were ordered in parallel and 8000 in antiparallel arrangement. In the parallel setup, a GrBP5-WT rotamer was just duplicated and translated, while the antiparallel configuration involved, after the translation, a 180° rotation and reflection about the plane defined by the GrBP5-WT backbone and vertical axis. Finally, each unit cell was randomly positioned on a bilayer HOPG supercell and underwent geometry optimization. This was carried out in ASE⁹⁰ using the BFGS optimizer, a stopping force threshold of $0.05 \text{ eV \AA}^{-1}$, and the previously trained NequIP NNP as a calculator. After the relaxation, about 26% of the unit cells became “asymmetrical” and were thus excluded from

further consideration. This asymmetry manifested in two ways: excessive misalignment of peptide backbones within a pair (which would conflict with the symmetry of the experimentally observed hydration layers), or the impossibility to replicate the relaxed unit cell into a supercell without significant padding between the replicas to avoid steric clashes. The remaining unit cells were then ranked based on how closely their geometry (i.e., their length, width and angle) matched with the experimentally measured one. The best ones resulting from this ranking were then assembled into supercells and visually compared to the 3D-AFM hydration layers, searching for pattern similarities. Among them, one structure appeared to match particularly well, displaying analogous topographic features both along the peptide nanowires direction and orthogonally to it, and was thus considered for further analysis of its hydration layers.

MD solvation structure

To simulate the solvation structure of the system we utilized classical MD, since NNPs are too computationally expensive to be used in ns production runs containing tens of thousands of water molecules. Thus, a 6×5 supercell of the best candidate deriving from the structure search was solvated, and its hydration structure was determined with MD. During the MD run of the system, which contained a total of 281,082 atoms, water molecules were allowed to move freely, but harmonic restraints were applied to the peptides and HOPG positions. The restraints were necessary since the potential energy surfaces of the NNP and MD FF are different, and, especially for a complex system like this one, this difference can lead to significant structural changes. The simulation was carried out in GROMACS⁹¹, parametrizing the peptides with CHARMM36⁹² and water with TIP3P. A 5 ns equilibration chain was followed by a 10 ns *NVT* production run, from which water oxygen density was calculated using MDAnalysis⁹³ with a 0.1 Å grid, sampling frames at 10 ps intervals. The H-bond analysis was also carried out using MD Analysis, defining bonds with a maximum water-oxygen to peptide-oxygen/nitrogen distance of 0.35 nm and a donor-hydrogen-acceptor angle of 100°.

Explicit tip free-energy simulations

A 3×3 supercell of the best candidate derived from the structure search was reparametrized using the OPC four-point water model⁷², which well reproduces the experimental bulk-water O–O radial distribution function including the second and third coordination shells. Peptides were parametrized using ff19SB⁹⁴ and water with the OPC parameter set, both via tleap, and converted to GROMACS format using ParmEd⁹⁵. The graphene-water non-bonded interaction was parametrized following Liao et al.⁹⁶, who derived explicit carbon-oxygen cross-interaction parameters for OPC water calibrated to reproduce the experimental graphite-water contact angle.

The AFM tip was constructed following Uhlig et al.⁹⁷, starting from a bulk diamond lattice with the [111] direction along the tip axis, from which a conical geometry was carved. Surface carbons bearing one dangling bond were passivated with hydroxyl groups, and those bearing two with two hydrogen atoms; the single apex carbon, bearing three dangling bonds, received a methyl group directed toward the sample and two hydrogen atoms on its remaining valences. The three hydroxyl groups immediately surrounding the apex conferred to the tip a hydrophilic character. The resulting tip model comprised 205 atoms (see Supplementary Notes 13 and 14). Force-field parameters were assigned under GAFF2, with atom types determined from local connectivity and partial charges derived at the AM1 semiempirical level using AMBER's sqm module, followed by tleap and ParmEd for topology construction.

A total of 18 positions were chosen on the peptide assembly plane (10 along the peptide aligned direction and 8 perpendicular to it), positioning the tip in each, resulting in 18 separate systems. Each system was energy-minimized, then equilibrated for 1 ns NPT

(Berendsen barostat, semi-isotropic, 1 bar) and 1 ns *NVT* (298 K, V-rescale, $\tau = 0.1$ ps), with full position restraints on graphene, peptide, and tip heavy atoms. Electrostatics used particle-mesh Ewald (fourth-order, 0.12 nm spacing) with a 1.0 nm real-space cutoff; Lennard-Jones used a 1.0 nm cutoff with potential-shift and an analytical dispersion correction to energy and pressure. Bonds to hydrogen were constrained with LINCS (order 4) under the Verlet scheme, enabling a 2 fs timestep, and graphene was an infinite periodic sheet. Production runs of 500 ns (*NVT*, timestep 2 fs, V-rescale thermostat, $\tau = 0.1$ ps) were carried out for each of the 18 equilibrated systems. During production, the apex methyl was unrestrained; the tip bulk was restrained in *x* and *y* ($K = 1000$ kJ mol⁻¹ nm⁻²) but free in *z*. To allow a more realistic tip-sample interaction, peptide restraints were softened near the tip according to *xy* radial distance: $K = 10$ kJ mol⁻¹ nm⁻² within 5 Å, linearly interpolated from 10 to 1000 between 5 and 8 Å, and 1000 beyond 8 Å.

Well-tempered metadynamics⁹⁸, as implemented in PLUMED 2.10⁹⁹ and coupled to GROMACS, was used to map the free energy along the tip-surface separation. The collective variable was the *z* component of the distance between the tip center of mass and a fixed reference atom at the mean graphene height, evaluated without periodic wrapping (PLUMED DISTANCE with COMPONENTS, NOPBC). This avoids the sign flips that POSITION would introduce when the tip crosses half the box length in *z*. Hills were deposited every 1 ps, with metadynamics parameters set to HEIGHT 0.50 kJ mol⁻¹, SIGMA 0.050 nm, BIASFACTOR 15. Harmonic walls ($KAPPA = 5000$ kJ mol⁻¹ nm⁻²) confined the CV between a lower bound placed 5 Å below the local peptide surface height and an upper bound of 4.0 nm, both expressed relative to the graphene reference plane.

Free-energy surfaces were reconstructed from the full 500 ns production trajectories, and each profile was shifted so that its minimum was set to zero. Convergence was assessed from the stability of the reconstructed profile over time, diffusive sampling of the CV across its range, and the slowing growth of the reweighting factor $c(t)$.

Statistics and reproducibility

To assess reproducibility, measurements were performed on 13 independently acquired peptide-water datasets and 15 peptide-in-PBS datasets. In total, 75 3D-AFM maps were recorded in water and 25 3D-AFM maps were recorded in PBS. For the analyses exemplified in Fig. 5, ≈ 200 individual Δf -*z* curves extracted from 2D maps were analyzed to determine the hydration-layer extent and the interlayer spacings (d_1 , d_2 , and d_3). The reported mean values and standard deviations were calculated from the distributions obtained from the individual curves. Interlayer spacings were included only when the corresponding hydration-layer features could be identified unambiguously. In addition, 24 individual Δf -*z* curves acquired without spatial averaging were analyzed to evaluate the local heterogeneity of the first hydration-layer spacing (d_1), see Supplementary Fig.17,f, i.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study, including the pre-trained model weights, training configuration file, training log, and training dataset, are available from Zenodo¹⁰⁰ and from the corresponding authors upon request. Unprocessed raw data, including the high-resolution AFM images, are provided as Supplementary Data 1, 2. Source data are provided with this paper.

Code availability

Code used in this study are available from Zenodo¹⁰⁰ and from the corresponding authors upon request.

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Author contributions

A.Y., L.S., M.S., and T.F. conceptualized the project. A.Y. performed AFM experiments and wrote the first draft. A.Y., L.S., K.H., T.S., and M.S. conducted data analysis. F.P. and A.S.F. performed simulations. L.P. and D.E.C. prepared the carbon tips. K.H., K. Miyazawa, and K. Miyata developed the analysis software. A.Y. and M.S. completed the editing and preparation of the final version of the manuscript. All authors contributed to the discussion of the results.

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Competing interests

The authors declare no competing interests.

Additional information

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