Hybridization and self-assembly behaviors of surface-immobilized DNA in close proximity: A single-molecule perspective

Qufei Gu1 | Eric A. Josephs2 | Tao Ye1,3

1 Department of Materials and Biomaterials Science and Engineering, University of California, Merced, California, USA
2 Department of Nanoscience, University of North Carolina at Greensboro, Greensboro, North Carolina, USA
3 Department of Chemistry & Biochemistry, University of California, Merced, California, USA

Abstract
Solid surfaces that are immobilized with DNA molecules underlie an array of biotechnological devices. These surfaces may also mediate the self-assembly of hierarchical DNA nanostructures. However, a number of fundamental questions concerning the structure–function relationship of these biointerfaces remain, including how these DNA probe molecules organize on the surface and how the spatial organization influences molecular recognition kinetics and interfacial affinity of these DNA molecules at the regime where crowding interactions are important (1–10 nm). This mini-review covers recent advances in understanding this structure–function relationship by spatially resolving surface hybridization events at the single-molecule level. Counterintuitive cooperative effects in surface hybridization are discussed and as is how modeling these cooperative effects can be used to predict the hybridization kinetics of a prototypical DNA sensor. Future opportunities in using mechanistic understanding to improve the performance and reliability of DNA sensors and form hierarchical supramolecular structures are also discussed.

KEYWORDS
atomic force microscopy, interfacial molecular recognition, molecular crowding, spatial distribution, surface patterning

1 INTRODUCTION
Surface hybridization, in which surface-anchored nucleic acid probes recognize and bind to their complementary targets in solution, is a fundamental process in a variety of biotechnological devices, including microarrays and DNA/RNA biosensors.[1,2] Apart from biosensing applications, surface hybridization also opens new avenues for supramolecular self-assembly and surface patterning.[3–5] A well-known challenge is that these devices often have notable platform-to-platform, batch-to-batch, and even device-to-device variations in performance.[6–8] While the exact origins of these variabilities remain unclear, they are fundamentally connected to the structure of the biointerfaces and the resulting complex interactions that influence both molecular recognition and signal transduction. At the interface, the interactions experienced by DNA probes as well as target molecules are substantially different from their counterparts in dilute solutions, where the thermodynamics and kinetics of molecular recognition are relatively well established.[9–13] Significant efforts have been made to explore how different properties of the biointerface, including probe design,[14,15] probe surface density,[16–20] surface chemistry,[21–24] and surface morphology,[25,26] affect the ability of DNA probes to recognize their targets. For example, using techniques such as surface plasmon resonance (SPR) that can quantify the overall amount of DNA probes at an interface, it was found that as the surface density of the DNA increases, their ability to hybridize with target molecules is hindered.[18,27,28] It is commonly assumed as the surface is packed with more probe molecules, these probe molecules become less accessible to target molecules due to electrostatic repulsion or steric hindrance. However, a lot of variabilities remain to be fully accounted for and there is indirect evidence that the impacts of heterogeneous spatial organization of probe molecules at the molecular scale,[29] which remains often poorly controlled and uncharacterized, may be profound: such heterogeneity in how probe
molecules are distributed on a surface may not only limit detection sensitivity \cite{30-32} but also be the root cause of the device-to-sensitivity variability \cite{4,7,33-35} found across many of these surface-based sensors and other devices. It is possible that even for surfaces with the same overall probe density, the molecular-scale crowding interactions experienced by individual probe molecules can be substantially different and their associated molecular recognition behaviors will diverge.

So far, the vast majority of our knowledge of these biointerfaces has been derived from measurements using techniques that average over many molecules on a relatively large surface area, such as SPR \cite{18,27,28} X-ray photoelectron spectroscopy (XPS) \cite{36-38} and electrochemical techniques \cite{1} and so the extent and effects of the molecular-scale heterogeneities are not well understood. The experimental observables from these techniques, such as average probe density and probe orientation, may not be adequate to describe the complex structures and interactions of realistic surfaces, which may have significant heterogeneity in probe densities and orientations. Indeed, spatially resolved fluorescence measurements of surface-immobilized DNAs revealed that significant heterogeneity exists even at the micron scale. \cite{39,40} However, to elucidate the roles of crowding interactions on such a heterogeneous surface, one needs to address the following questions. First, where exactly are each of the probe molecules on the surface? Their lateral arrangement is a key aspect of the interfacial structure that fundamentally determines the crowding interactions. Second, where do target molecules bind? Do they bind randomly, or do they bind preferentially to specific probe molecules? Three, how do these interactions ultimately impact device performance? Unfortunately, these seemly simple questions concerning molecular recognition at biointerfaces have in fact been prohibitively difficult to answer. The crowding interactions are typically significant when these molecules are separated by approximately 10 nm or less, which is beyond the resolving power of common microscopy techniques. Without the ability to address these three key questions, our knowledge of the structure–function relationship for DNA biosensors and microarrays would remain very limited.

Although studies based on results from ensemble-averaging techniques assumed that (1) the DNA probe molecules are uniformly distributed across the biosensor surface, (2) they display similar hybridization kinetics, (3) crowding interactions are insignificant if their surface density is below $10^{12}$/cm$^2$ which corresponds to an average inter-probe distance of 10 nm, and (4) crowding interactions impede hybridization. Results from more recent microscopy studies \cite{48,49,50-52} especially those based on single-molecule atomic force microscopy (AFM) imaging of model electrochemical DNA (E-DNA) sensors \cite{41,62} show that these assumptions may need to be revisited. Heterogeneity appears to be particularly important for surface-immobilized DNA probes even when their spatial distribution is mostly random. The commonly used hairpin DNA probes may exhibit cooperative behaviors in target binding when they are in spatial proximity. Beyond detection of biomarkers, surface hybridization can mediate the formation of DNA-based biomaterials at the surface as surface hybridization can in principle be extended to branched DNAs such as DNA origami.

The goal of this mini-review is to review recent advances in the understanding of the effect of crowding interactions on interfacial molecular recognition from a single-molecule perspective. This review first provides a brief overview of techniques being used in characterizing surface-immobilized DNA and the challenges in elucidating crowding interactions. The review then covers the strategies being employed to map the surface-immobilized DNA molecules as well as spatial statistical tools to describe the spatial distribution. It then examines the effect of crowding interactions on interfacial molecular recognition, which has major impacts on not only biosensing function but also supramolecular assembly at surfaces. While there are a number of excellent reviews addressing the characterization and properties of surface-immobilized nucleic acids \cite{2,33,46} this review explores how crowding interactions can exert a major influence on interfacial molecular recognition from a single-molecule perspective, which requires spatially mapping individual DNA probe molecules with sub-10 nm separation. A few studies that resolved single-molecule hybridization event relies on extremely diluted DNA coverages where crowding interactions were insignificant due to limited resolution.

## 2 | CHARACTERIZATION OF SURFACE-IMMobilIZED DNA

### 2.1 | DNA surface immobilization

DNA biosensors and microarrays typically require the immobilization of the DNA probe onto a solid support because the solid support enables a variety of mechanisms that transduce molecular recognition events into detectable signals. \cite{2} DNA immobilization has been investigated on various substrates (planar or nanostructured surfaces) such as glass, silicon, carbon, polymer, and gold with distinct chemical or physical properties. \cite{1} Moreover, the site-specific immobilization of DNA sequences enables multiplex detection of many different biomarkers. One of the examples is the DNA microarray that allows the parallel detection of thousands of analytes via spatially resolved immobilization. \cite{147} One way to construct such patterned DNA arrays is the direct production of probes on the surface using light-directed synthesis of oligonucleotides \cite{48,49} which uses light to deprotect the surface-anchored strands and couple new nucleotides in a stepwise and spatially addressable fashion. While light-directed synthesis is ideally suited for patterning a larger number of DNA sequences, the error rate of the DNA sequence is relatively high, limiting this technique to short oligonucleotides. Alternatively, pre-synthesized DNAs can be site-specifically immobilized on cationic solid supports through droplet deposition \cite{50} or microfluidics. \cite{51} While this immobilization approach is easy to implement, the DNA probes are likely adsorbed onto the surface in the “loop-trains-tails” configuration, where a single polymer chain has multiple adsorption sites along the surface, \cite{52} adversely impacting their ability to recognize DNA targets. \cite{53} Hence end tethering of the capture probe to the surface commonly used in many DNA biosensors. \cite{1} This is often realized by covalent coupling of chemically modified probes to a preassembled self-assembled monolayer \cite{54} or attachment of thiolated probes to gold surfaces via Au–S bonds. \cite{17}
2.2 | Surface characterization methods

Although ex situ analysis methods such as XPS,[36–38] ellipsometry,[55] and secondary-ion mass spectrometry (SIMS)[38] can provide insight into the chemical, structural, or morphological properties of surfaces, they are limited by the need to remove samples from their native environments. Here we focus on methods that characterize surface-immobilized DNA. The usage and limitations of spectroscopic (infrared (IR), SPR, surface-enhanced Raman spectroscopy (SERS), neutron and X-ray reflectivity), mechanical (quartz crystal microbalance (QCM)), electrochemical (cyclic voltammetry (CV), differential pulse voltammetry (DPV), square-wave voltammetry (SWV), chronocoulometry, electrochemical impedance spectroscopy (EIS)), and fluorescence microscopy techniques are discussed below.

Spectroscopic techniques such as IR spectroscopy[46,56–58] and SERS[59–62] can provide molecular-specific information such as the orientation of the adsorbed species and the composition of the interface. However, the spatial resolution is typically limited to a few micrometers due to the diffraction limit. While SERS exhibits significantly improved sensitivity,[59–62] the interpretation of SERS results is complicated by an overwhelming of the signal from “hotspots” located at the sharp corners and edges of localized nanostructures. By measuring the depth profiles of thin films, neutron reflectivity (NR)[63] can be used to characterize the thickness, density, and orientation of immobilized DNA layers. Through monitoring the amount of materials adsorbed at the surface, SPR[64–68] and QCM[69–72] are suitable for probing immobilization and target binding kinetics in real time and a label-free manner. However, these techniques do not discriminate between the types of molecules it detects. Notably, although recent advances in SPR imaging have enabled imaging and tracking of single DNA molecules, single-molecule imaging was only achieved on surfaces with extremely dilute coverages where crowding interactions were insignificant.[73–75]

Electroanalytical methods such as voltammetry,[76,77] and coulometry[78,79] measure the electron transfer between the redox active moieties on the DNA probes/targets and the electrode surface to characterize the surface-immobilized DNA.[11] While earlier studies utilized the direct electrooxidation of the guanine bases,[80,81] electrochemical characterization of DNA typically relies on redox active labels that either are conjugated to or intercalate with DNA. The redox labels enhance the signal, improve selectivity, and enable various signal transduction mechanisms for detection. Different waveforms give rise to many forms of voltammetry, such as CV, DPV,[76,77] and SWV.[82–86] On the other hand, EIS determines the impedance of the electrode surface as a function of frequency, which is sensitive to the variation in interfacial properties caused by target binding.[87–89] As the Faradaic impedance is a function of electron transfer kinetics, crowded areas (e.g., aggregation of immobilized probes) with high impedance could impede or block electron transport. A major appeal of these electrochemical methods is that they cannot only be used to characterize the surface immobilization of DNA probes but also serve as practical signal transduction mechanisms to detect the binding of target molecules to these DNA probes, as target binding often alters the electrochemical signal by changing the distance between the electroactive species and the electrode surface[90] or the amount of electroactive species close to the electrode surface.[91–93]

Fluorescence spectroscopy/microscopy has been applied to examine the heterogeneity and density of the DNA probe surfaces with different degrees of crowedness. For example, the distant-dependent quenching of fluorescence can be used to monitor the dynamic orientational switching of fluorophore labeled DNA tethered to a gold electrode when the electrochemical potential is changed.[94] In addition, fluorescence microscopy was used by Bizzotto and coworkers[39,40] to monitor the orientation switching in a spatially resolved manner. Although these studies revealed surface heterogeneity and aggregation at the micron scale, the spatial organization of single DNA molecules was not determined and an quantitative understanding of the effect of molecular crowding was unavailable. Total internal reflection fluorescence microscopy (TIRF), when coupled with intermolecular resonance energy transfer, has successfully detected hybridization of single DNA molecules.[43] However, single-molecule imaging was only achieved on surfaces with extremely dilute coverages (<10^10 probes/cm^2) where crowding interactions were insignificant. Although single-molecule force spectroscopy (SMFS) by atomic force microscope can differentiate single-stranded DNAs (ssDNA) from hybridized double-stranded DNAs (dsDNA) at nanometer scale,[95–97] the spatial resolution of 30–100 nm remains inadequate for single-molecule characterization of crowding interactions where the DNA molecules are separated by approximately 10 nm or less (Figure 1A).

The discussion above shows while existing characterization techniques can provide valuable information concerning the immobilized DNA probes as well as the binding of targets, they are typically restricted to reporting characteristics of the biosensor surface averaged over an area that is at least microns in size. Therefore, basic questions concerning the biointerface, such as the spatial arrangement of surface-immobilized DNA and how it influences surface hybridization have remained unanswered. Due to the limited information obtained from current experimental techniques, existing theoretical models to describe crowding effects[27,98] had to rely on the assumption that the DNA molecules are uniformly distributed.

3 | SINGLE-MOLECULE AFM IMAGING OF DNA IMMOBILIZED ON GOLD

3.1 | How to observe individual DNA probe molecules?

The discussion above underscores the need for mapping the individual surface-immobilized DNA molecules even when they are separated by 10 nm or less. We have found AFM to be ideal for this purpose. By monitoring the interactions between a very sharp tip of silicon “probe” stylus and a surface as the stylus is rastered, a nanometer scale, real-space image of a sensor surface can be reconstructed in a way that permits the direct resolution of the location, conformation, and hybridization status of individual DNA molecules on the surface.[14,45] To image DNA molecules under conditions that are relevant to the operation of the sensors, AFM imaging can be performed while both the sample and stylus are submerged in a buffered aqueous solution. Additionally, to minimize significant molecular perturbation, or displacement of the molecules at the surface by the stylus, which
can degrade imaging resolution, scanning is typically performed under the “intermittent contact” or “tapping mode” where the stylus is oscillated near its resonance frequency: as interactions between the tip and sample change the phase or amplitude of the oscillating stylus, and the surface can then be mapped from those less-perturbing interactions.\(^{[99]}\)

In addition to imaging under less-perturbing conditions, two other requirements are important for spatially resolving DNA probes as well as targets that have hybridized with the DNA probes: the atomic scale flatness of the surface and interactions that pin DNA to the surface. Commonly used gold surfaces such as gold thin films evaporated onto silicon, have atomic-level roughness of a gold surface (evaporated onto silicon, for example), significant roughness of the gold makes individual DNA molecules otherwise difficult to discern. In our studies, atomically smooth gold Au(111) crystalline surfaces were prepared as model sensor surfaces.\(^{[29,44,100]}\) These surfaces allow us to elucidate the impacts of spatial organization of DNA probes with minimized interference by other factors such as morphological heterogeneities. We have demonstrated that electroactive DNA tethered to these atomically smooth gold substrates can transduce target binding into detectable electrochemical signals, making these surfaces function as biosensors that can detect DNA targets.\(^{[41,42]}\)

In addition, AFM can only clearly resolve a molecule when it is immobile during the time scale of scanning.\(^{[44,99]}\) Although the DNA probe molecules are “immobilized” onto a gold surface through end tethering, these end-tethered molecules remain too mobile for high-resolution AFM as they may rotate or deflect in response to perturbation from an AFM tip. The dilemma is that if the molecules are strongly pinned to the surface, they are not capable of binding to their target molecules in solution, which would limit our ability to study molecular recognition. To address the challenge, we developed two different approaches that allowed us to switch surface interactions: either pinning the DNA molecules tightly to the surface so that they could be efficiently imaged and resolved by AFM or releasing the free end of the DNA molecule to interact with targets in the solution.\(^{[44]}\) These immobilization techniques exploit the negative charges of the DNA phosphate backbone, such that by introducing positive charges to the surface, either potentiodynamically\(^{[100]}\) or by changing the composition of the solution, the DNA can be pinned to the surface.\(^{[44]}\) The DNA molecules could be reversibly immobilized to the surface for imaging at a positive potential, or released into the solution when the electrode potential is below the potential of zero charge (Figure 1B). The potentiodynamic method allowed us to switch interactions rapidly and allowed us to investigate the effects of heterogeneities on the interactions between the probe DNA molecules and the surface.\(^{[100]}\) However, the strength of immobilization remains insufficient for clearly resolving ssDNA probes and may irreversible damage and morphology changes occurred on the surface monolayers after repeated potentiodynamic cycling.\(^{[45]}\) To allow us to spatially resolve surface hybridization of single DNA molecules, the surface interactions were reversibly tuned by changing the composition of the buffer.\(^{[44]}\) In a monovalent cation buffer, the end-tethered DNA is repelled by the
The nanoscale spatial distributions of DNA probe molecules are highly sensitive to sensor preparation method

Different spectroscopic, microscopic, mechanical, and electrochemical techniques have revealed that the distribution and reactivity of DNA molecule on a sensor exhibit sensitivity to their preparation and immobilization method.\(^{[17,33,39,105]}\) and using AFM we have been able to resolve molecular-level structural characteristics that can provide insights into these behaviors. One critical requirement for DNA sensors is surface passivation. Non-specific and hydrophobic interactions between ssDNA and a gold surface can be significant and will pin ssDNA probes to the surface in a way that will prevent additional probes from binding to the surface and also inhibit recognition of target molecules in solution. Hence to reduce non-specific interactions between the gold surface and the probe/target molecules and avoid overcrowding of the surface with DNA probe molecules, thioldated DNA probes are often immobilized on a sensor surface in the presence of a co-adsorbate, typically a short hydrophilic alkanethiol, such as 6-mercaptohexanol. This passivation can be performed by simultaneous exposure of a solution containing the thioldated DNA and hydrophilic alkanethiol to the surface (co-adsorption) or by sequential exposure, first to the thioldated DNA probe then to the hydrophilic alkanethiol (the “backfilling” method, left panel in Figure 2A) whose own Au–S interactions will displace non-specifically adsorbed ssDNA.\(^{[117]}\) In both cases, AFM imaging showed that rather than resulting in a uniform monolayer of DNA probes interspersed among the hydrophilic alkaneithiols that had previously been assumed, these sensor preparation methods result in nanoscale segregation of the molecules to regions of high and low densities of DNA probes (right panel in Figure 2A), as both the alkaneithiols and the DNA probes have migrated, leading to phase separation.

Taking inspiration from the field of molecular electronics, where methods to disperse isolated molecules of interest on a surface had been previously developed by Weiss and coworkers,\(^{[102]}\) we then determined the structure of a sensor surface prepared by first assembling a monolayer of the short hydrophilic alkanethiol on the gold surface, then exposing the surface to thioldated DNA probes (the “insertion” method, left panel in Figure 2B). Short alkaneithiols (4–16 carbon long) are known to form ordered monolayers on Au(111) surfaces with nanoscale crystalline domains, with discontinuities, holes, and defects in the monolayers at the location of gold adatoms or adlayers and alkanethiol domain boundaries. These defects in the alkanethiol monolayer, can provide sites for other thioldated molecules to attach to the surface. We found that the assembly of DNA probes using the “insertion method” resulted in surfaces with isolated, individually resolvable probe DNA molecules with spacing of sub-10 nm to tens of nanometers (right panel in Figure 2B), depending on exposure conditions, length of alkanethiol adsorbate, probe structure, and the length of alkanethiol attached to the DNA probe.\(^{[29,45]}\) The distribution of the DNA probes on the surface assembled in this way exhibited significant spatial randomness instead of a regular or periodic pattern. Therefore, we found the commonly used average distance calculated from the probe surface density: \((A/n)^{1/2},\) which assumes that the molecules are arranged in a square lattice (A is the area of the surface and n is the number of molecules),\(^{[19]}\) substantially overestimates the actual mean nearest neighbor distance (NND) and hence underestimates the crowding interactions.\(^{[29]}\)

As statistical methods more suited to this task had been developed, for example, in fields of forest ecology to describe the geographic position of trees, we defined a crowding function \(N_i(r)\) and related metrics for nanoscale DNA probe crowding known as the “local crowding index” (LCI) based on Ripley’s K function \(K(r)\).\(^{[105]}\) which is used in spatial statistics to quantify the aggregation or dispersion of objects at short- and long-range scales (Figure 2C). The crowding function \(N_i(r)\) is defined as the number of surrounding probe DNA molecules within distance \(r\) of probe \(i\), and the distribution of \(N_i(r)\) for a given sample can reveal interesting descriptive features about the level of heterogeneity of probe molecule crowding at the nanoscale as a function of different
preparation methods across different scales of molecular organization, and the normalized average of which converges to Ripley’s $K$ function can be used to perform rigorous tests of spatial statistical properties. The LCI is defined as the crowding function or number of neighboring probes within the maximum distance across which molecular interactions could be expected to occur and potentially affect probe–target interactions: we initially hypothesized an LCI defined by a distance of twice the hybridized probe length could sufficiently describe the effects of probe crowding on target binding, although later we left this as a free parameter that can be estimated from the observations of probe–target binding rates as a function of probe crowding. These methods allowed us to quantitatively define how sensor preparation could be used to influence the nanoscale distribution and aggregation of probes molecules on a surface, with the ultimate aim of reproducibly controlling this aspect to make device performance more reliable.

### 3.3 Direct patterning of single-probe molecules using an atomic force microscope

Because during AFM imaging the structure of a surface is determined by maintaining controlled mechanical interactions between the stylus tip and the surface, by increasing the interactions between the tip and the surface so the two come into direct contact for sustained intervals, the AFM tip can mechanically deform portions of the surface in a predefined way and generate nanoscale patterns. This process of mechanical deforming surface structures has been used to mechanically desorb nanoscale regions of preformed alkanethiol monolayers on gold surfaces and, when performed in the presence of another alkanethiol molecule in solution, that second molecule will assemble in the newly exposed region in a process known as “nanografting.”[104,105] Nanografting has been performed using thiolated DNA molecules in solution to assemble nanoscale patterns of ssDNA probes on a surface able to react with target DNA molecules in solution. The nanografting process results in chemical patterns with interesting properties. For example, if nanografting is performed in the presence of two different alkanethiols, the resulting pattern will be significantly more well mixed than those mixed monolayers assembled directly onto a gold surface that can form large segregated domains. Exploiting this property, we also found that by optimizing the ratio of thiolated DNA and a co-adsorbate in solution during nanografting, individual DNA probe molecules could be directly positioned into nanoscale chemical patterns with a relatively high yield ($\sim60\%–70\%$, Figure 3A–C)[106] which could subsequently be induced to interact with target and probe molecules (Figure 3D,E)[107] and nanografting provided a reliable method to introduce isolated DNA probe molecules with well-defined nanoscale chemical environments onto sensor surfaces, albeit for small-scale studies using only tens to dozens of probes.[107]

### 4 EFFECT OF CROWDING INTERACTIONS ON INTERFACIAL MOLECULAR RECOGNITION

#### 4.1 How do crowding interactions affect target binding?

With the ability to spatially resolve DNA probes as well as DNA targets that bind to the DNA probes, one can begin to address this question: where do target molecules bind? Our model system is electrochemical DNA (E-DNA) sensors, which were pioneered by Plaxco and coworkers.[108] E-DNA sensors utilize the conformational changes of surface-immobilized electroactive DNA probes, typically stem-loop (hairpin) DNA probes, upon target binding to detect analytes. Analogous sensors that use DNA aptamers
as the probes have also been developed to detect small molecules as well as proteins.[] These sensors hold significant potential in point-of-care diagnostics due to their abilities in highly selective, label-free, and miniaturized detection of a range of biomarkers in complex biofluids. Although E-DNA sensors use end tethering of thiol groups to immobilize DNA probes onto gold electrodes, which should yield more controlled conformations than other immobilization methods,[] reproducibility remains a significant concern. We constructed model surfaces that serve as E-DNA sensors and simultaneously enabled AFM to spatially resolve surface hybridization even when the inter-probe separation is less than 10 nm, allowing us to probe the regime where crowding interactions are important.[42] The surfaces, which consist of electroactive DNA probes tethered to highly ordered self-assembled monolayers (SAMs) on an atomically flat gold electrode, allowed us to perform both ensemble electrochemical target detection as well as single-molecule AFM imaging. The model surface presents a wildly varying spatial distribution of probe molecules (left panel of Figure 4A), the characterization of which was not accessible by ensemble averaging or common single-molecule techniques. The NND (the distance between a probe and its closest neighboring probe) of each of the probe molecules was displayed in the heat map in the right panel of Figure 4A. Notably, the broad distribution of NND values ranging from a few nanometers to as many as 60 nm is a consequence of random distribution instead of clustering or repulsion of molecules, revealed by Ripley’s K function analysis.[42] Given the spread in NNDs, one might expect that the target molecules would be less likely to bind to probe molecules with smaller NNDs as the crowding interactions should in principle make the probe molecules less accessible to target molecules, as shown in previous studies that use ensemble-averaging techniques to study the impact of DNA probe crowding.[17–19,27,28,112] Instead, the opposite was observed by AFM: the target molecules predominantly bind to probes in crowded regions where the NND is less than 15 nm (red dots in the right panel of Figure 4A), suggesting that the presence of neighboring probes in proximity may accelerate the hybridization kinetics. This observation is further supported by two statistical analyses: (1) nearly 70% of the probes with NND of 10 nm are hybridized after 105 min of exposure to their targets, while the fraction of hybridized probes with NND of 30 nm is only 5% (Figure 4B); (2) for probe molecules with the same NND, those with higher LCI (number of neighboring probes surrounding a particular probe) are more likely to capture target (Figure 4C). Our control experiment done with linear DNA probes did not display preferential binding to crowded regions, suggesting that the secondary structure of the DNA hairpin probe in E-DNA sensors is responsible for this counterintuitive trend (Figure 4B,C). We proposed three possible mechanisms. First, electrostatic repulsion arising from folded neighboring probes may promote target binding by destabilizing the stem-loop structure[113] (Figure 4D). Although the DNA hairpin structure is often used to improve selectivity and enable signal transduction, the hybridization with DNA hairpin probes is slower than hybridization with linear probes due to the need to melt the stem. By destabilizing the folded state, the kinetic barrier can be reduced and hybridization accelerated. Second, neighboring probe molecules in close proximity can trap target molecules and increase their residence time (Figure 4E). Many unstable/unproductive contacts between the target and the stem-loop probe predate the formation of a full target–probe duplex. A transiently bound target hopping onto neighboring probe may offer prolonged residence time and enhanced accessibility to probe molecules. Third, once a target molecule binds to a probe, the neighboring probe molecules experience crowding interactions that also accelerate target binding. Studies are underway to understand the relative contributions from these mechanisms. Another question is how to reconcile our findings and the prevailing assumption that surface crowding hinders target recognition and optimal hybridization kinetics is achieved at the lowest densities.[17–19,27,28,112] Two factors could be responsible. First, as existing studies used ensemble-averaging techniques to characterize the target binding of surfaces that have highly heterogeneous inter-probe separations,[29,34,39] it was not feasible to quantify the influences of specific inter-probe separations. Our spatially resolved, single-molecule characterization of hybridization represents an ideal way to address this question. Second, many existing studies focus on high surface density regimes, 10^{12}/cm^{2} or greater,[18,27,112] where the accessibility

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**FIGURE 3** Schematic illustration of nanografting surface chemical patterns with DNA seed strands and capture of DNA targets. (A) A large force was applied on the atomic force microscope (AFM) tip to displace the thiol molecules in the MHDA (16-Mercaptohexadecanoic acid) self-assembled monolayer (SAM) on an Au(111) substrate. (B) The thiol molecules in the solution, 24 nt single-stranded DNA (ssDNA) with C_{11}H_{22}SH tethers and 11-mercaptoundecanoic acid (MUDA) form a mixed monolayer on the exposed gold surface. (C) An AFM image of a patterned single-molecule array. (D) The ssDNA molecules can capture double-stranded DNA with a single-stranded segment. (E) An AFM image of nanoarray with captured targets. Imaging was carried out in a Ni^{2+} buffer that immobilized the DNA molecules. Scale bars are 200 nm. Reproduced with permission.[107] Copyright 2013, Royal Society of Chemistry.
to probe molecules may indeed be the limiting factor. Our study focuses on a less crowded regime ($\sim 10^{11}/\text{cm}^2$), where accessibility is less likely a bottleneck and mechanisms for enhanced hybridization can manifest themselves.

4.2 How does the spatial organization of probe molecules influence the hybridization kinetics?

The results in the preceding section provide direct evidence that crowding interactions can alter target binding in unexpected ways. While the target binding kinetics at the single-molecule level provides novel insight into surface hybridization, ultimately we need to understand how the lateral organization of probes influences the overall hybridization kinetics of a sensor surface. As the overall hybridization kinetics determines several key performance metrics of the DNA sensor, such as limit of detection and detection speed, such a structure–function relationship is critically needed for rational engineering of DNA-based sensors and microarrays.

We investigated whether spatial statistical models of single-molecule rate constants extracted from spatially resolved DNA hybridization can predict the overall binding kinetics (Figure 5A).\(^\text{[41]}\) We first categorized the probe molecules based on their NNDs and LCIs and extracted the corresponding rate constants from the first-order Langmuir fitting of the kinetic curves, which are displayed as green dots in Figure 5B. We found that the highest rate was observed for those probes with a high LCI and a low NND, further confirming that the presence of neighboring probe molecules may accelerate target binding. To model the single-molecule rate constant, we applied Cox proportional hazard regression (pink surface in Figure 5B) using the following equation:

$$k_i = k_0 \exp\left(\alpha_{\text{LCI}i} \cdot \text{LCI}_i + \beta_{\text{NND}i} \cdot \text{NND}_i\right)$$

(1)

where $\alpha_{\text{LCI}i}$ and $\beta_{\text{NND}i}$ are regression parameters related to LCI and NND and $k_0$ is the baseline rate constant. To connect these single-molecule behaviors in a heterogeneous local environment to the overall hybridization performance and test the validity of this model, we incorporated the predicted single-molecule rate constants into numerical simulation that uses average rate constants to regenerate the overall kinetic traces on E-DNA sensor surfaces and establish the model of single-molecule rate constants. The divergent kinetics of E-DNA sensor surfaces with similar average surface densities but different degrees of probe aggregations (Figure 5C) were reproduced. This offers direct evidence that the spatial arrangement of probe molecules is a major determinant of the sensor performance and the widely used average probe densities\(^\text{[16–19]}\) do not serve as a reliable indicator of the crowding interactions. Moreover, the facile hybridization of E-DNA sensor surface with some degree of probe clustering (purple curves in Figure 5C) indicates that, under certain conditions, crowding interactions can accelerate target recognition of hairpin probes. Interestingly, the simulated traces (E-DNA sensor surface that are not used to build the model of single-molecule rate constants, purple solid curve in Figure 5C) were able to predict the major features of the experimental traces (purple dashed curve in Figure 5C), highlighting the utility of our models that can account for heterogeneity in surface hybridization.
FIGURE 5  Quantitative modeling and prediction of hybridization kinetics at the crowded interface. (A) Schematic of a predictive model of single-molecule rate constant based on spatial statistics. (B) Cox proportional hazard fitting (pink surface) of the single-molecule rate constants (green dots). (C) Numerical simulation (purple and black solid lines) reproduced the divergent hybridization kinetics of two surfaces with similar overall surface densities, but different spatial patterns of probe molecules (purple and black dashed lines). Reproduced with permission. [41] Copyright 2021, American Chemical Society

FIGURE 6  DNA origami self-assembly at the crowded interface. (A) Schematic of surface-mediated self-assembly of DNA origami and representative atomic force microscopy (AFM) images at different stages of the self-assembly process. (B1 and B2) AFM images of scaffolds folded by staples in a buffer containing 12 mM Mg$^{2+}$ and a buffer containing 1 M NaAc, respectively. The scale bars are 50 nm. (C1 and C2) Aggregation of DNA origami structures induced by local crowding of tethered DNA scaffolds. The scale bars are 100 nm. (D) AFM image of surface-seeded growth of different DNA origami shapes on a surface that has two different seed strands. The scale bar is 100 nm. Reproduced with permission. [5] Copyright 2020, American Chemical Society

5 | SURFACE-SEEDED SELF-ASSEMBLY OF DNA ORIGAMI

In addition to biotechnological devices such as biosensors and microarrays, we have shown that surface hybridization may be exploited to initiate the self-assembly of complex supramolecular structures, such as DNA origami, at surfaces (Figure 6A). [5] Pioneered by Rothemund, [114] DNA origami can form a large variety of nanoscale shapes by hybridizing a long ssDNA (scaffold) with a collection of synthetic oligonucleotides (staples) that are complementary to specific regions on the scaffold. Due to the formation of designer nanoscale shapes as well as the ability to display other molecules and materials with nanometer precision, a large variety of applications of DNA origami are being explored, including nanoscale machines, drug delivery, nanosensors, nanophotonics, and nanoelectronics. [115] While these DNA origami structures are typically assembled in the solution phase, it is appealing to grow these structures at a surface as a separate deposition step is no longer needed for interfacing them with materials and the structures can potentially be grown site-specifically. While the surface hybridization of biosensors and microarrays involves linear DNAs, here the surface immobilized with DNA is mediating the self-assembly of DNA origami, that is, hybridization of branched DNAs. This approach allows us to investigate how crowding interactions affect complex multicomponent self-assembly, shifting from a “two-body” to a “many-body” system. Growing such structures at a solid–liquid interface has numerous advantages, such as...
AGGREGATE biointerface structure that may have significant influences on the interaction of these surface-immobilized DNA is a key aspect of the overall hybridization kinetics from the distribution of probe densities could have distinctly different hybridization efficiencies. Moreover, surfaces with similar lateral organization influences surface hybridization. Critical to the successful self-assembly is controlling the interactions between DNA and the surface (Figure 6B). DNA origami self-assembly, which involves hybridization of branched DNA, requires a salt to screen electrostatic repulsion between phosphate groups. While a divalent cation such as Mg$^{2+}$ is commonly used, Mg$^{2+}$ also mediates adhesion of the DNA to the carboxyl-terminated SAM surface, which not only reduces their conformational freedom for folding but also hides their binding sites for staples. In a solution with concentrated Na$^+$, under which the scaffolds only interact weakly with the surface, we found that these scaffolds are afforded the conformational freedom needed for self-assembly (Figure 6B). Akin to the aggregates formed by captured targets in Figure 4A, regions crowded with surface-tethered scaffolds produced aggregation of DNA origami due to blunt-end stacking (Figure 6C1) or possible DNA staple bridging between two neighboring scaffolds (blue arrows, Figure 6C2). Importantly, the same patterns of aggregation were not observed in the folding of untethered scaffolds in a homogeneous solution.

Our approach to growing supramolecular complexes at surfaces potentially offers a means of encoding information into the surface to control DNA self-assembly.[5] We have demonstrated that these surface-anchored strands can be used to capture specific DNA scaffolds that are later folded into different designer shapes (Figure 6D). Therefore, this approach allows us to sequence the number of seed strands to determine the size and shape of DNA origami to be grown site-specifically.

6 | CONCLUSIONS AND FUTURE DIRECTIONS

With the development of AFM imaging techniques to map single-probe molecules on model DNA biosensor surfaces and a spatial statistical framework to describe the spatial distribution of both probe and target molecules, new insights into how the lateral organization influences surface hybridization have emerged. While it has generally thought that crowding interactions inhibit target binding, our studies of DNA hairpin probes in spatial proximity showed a cooperative effect that enhances target binding. Moreover, surfaces with similar probe densities could have distinctly different hybridization kinetics, underscoring that the average probe density that has been used to characterized DNA biosensors may not be a reliable indicator to describe crowding interactions. Our studies also showed heterogeneity of target binding due to crowding interactions even on surfaces where the probes are mostly randomly distributed. Our preliminary successes in predicting overall hybridization kinetics from the distribution of probe molecules provide support that the lateral spatial organization of these surface-immobilized DNA is a key aspect of the biointerface structure that may have significant influences on interfacial molecular recognition.

These advances set the stage for future studies that will provide a detailed mechanistic understanding and more accurate prediction of both hybridization kinetics and device performance. Moreover, due to the limited imaging resolution, our studies focused on a relatively less crowded regime (10$^{11}$ probes/cm$^2$) where the underlying mechanisms of crowding interactions could deviate from those for biosensors with higher probe densities (>10$^{12}$ probes/cm$^2$). For the same reason, it is difficult to reliably incorporate the effect of the target-induced crowding and the unfolding of hairpin probes into the modeling of binding kinetics. The advancement of non-contact AFM that can potentially achieve sub-nanometer resolution can help us to uncover the hidden mechanisms on biosensor surfaces that are even more crowded with probe and target molecules. Our discoveries that the surface-anchored strands can seed the self-assembly of surface-anchored DNA origami and the surface-anchored DNA origami in spatial proximity may connect through blunt-end stacking interactions suggest new mechanisms to allow sophisticated information encoded into specific interactions at the surface to direct the self-assembly of complex surface architectures. One can envision top–down methods capable of patterning anchor strands to initiate the site-specific growth of tethered DNA origamis in spatial proximity, which will enable these structures to connect to form complex hierarchical structures that are difficult to form in the solution phase.

Our findings also raised the intriguing prospect that the lateral inter-probe spacing may be deliberately tailored to improve the performance of biosensors. In traditional DNA immobilization methods, the control over the spatial organization of probes remains limited,[16–19] which may not only reduce the detection sensitivity but also be the nanoscopic origin of the large device-to-device variabilities of existing biosensors. In a more recent study, we showed that DNA origami can serve as a nanoscale stamp that implants a dimer onto a passivated gold surface with a controlled inter-probe separation.[120] This opens the interesting prospect of predetermined specific inter-probe distances as well as geometrical arrangements of DNA probes at the biosensor surface. Therefore, one may have surfaces in which individual DNA probe molecules are immobilized with tailored spatial patterns that could improve the kinetics of target binding. In addition to surface hybridization, our approach of combining spatially resolved measurement on model surfaces with spatial statistical analysis may be applied to other types of molecular recognition, such as aptamer sensors that detect proteins,[109–111] as well as ligand–receptor interactions at cell membranes.[121,122] Furthermore, the underlying mechanisms for the usual aggregation of surface-folded DNA origami may provide new insights into two-dimensional positioning of functional elements and assembling of surface-confined supramolecular networks.[123] Therefore, probing and analyzing these biointerfaces at the molecular scale will lead to fundamental insights for both biotechnological devices and cell signaling processes.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Tao Ye https://orcid.org/0000-0001-8615-3275

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based biosensors behave at the molecular level and single-molecule techniques to elucidate how surface-
joined the faculty of University of California, Merced
laboratory at the Pennsylvania State University. He
biophysics and molecular bioengineering to develop safer
synthetic biology and uses approaches from molecular
Nanoscience in the Joint School of Nanoscience and
Carolina at Greensboro (UNCG) in the Department of
PhD in Biological Engineering & Small-Scale Technolo-
Dr. Tao Ye received his BS degree from Peking University, and then went to the University of Pittsburgh to pursue his graduate study under the direction of Eric Borguet and received his PhD in chemistry in 2003. He then undertook his postdoctoral training in the Paul Weiss laboratory at the Pennsylvania State University. He joined the faculty of University of California, Merced in 2007. His group has broad interest in biofunctional interfaces. He and his coworkers have developed novel single-molecule techniques to elucidate how surface-based biosensors behave at the molecular level and how DNA self-organize into complex nanostructures at surfaces.

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AUTHOR BIOGRAPHIES

Quefei Gu received his BS degree in Physics from Sichuan University in 2011. After obtaining his MS degree in Physics from the University of New Mexico in 2014, he went to the University of California, Merced to pursue his doctoral study under the direction of Tao Ye and received his PhD in Biological Engineering & Small-Scale Technologies in 2019. He is currently pursuing his postgraduate study under the direction of Zhiyong Wang at Carnegie Institute of Science, Stanford University. His research interests focus on biosensors, DNA nanotechnology, and nanoparticle-mediated gene editing and delivery.

Eric Josephs earned his PhD from the University of California, Merced in 2013 in the laboratory of Prof. Tao Ye. After additional postdoctoral training at Duke University in Durham, NC, in the laboratory of Prof. Piotr Marszałek, he joined the faculty of the University of North Carolina at Greensboro (UNCG) in the Department of Nanoscience in the Joint School of Nanoscience and Nanoengineering (JSNN). His laboratory has a focus on synthetic biology and uses approaches from molecular biophysics and molecular bioengineering to develop safer and more effective biotechnologies.