One-pot reaction for the preparation of biofunctionalized self-assembled monolayers on gold surfaces

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Abstract

The Huisgen cycloaddition reaction ("click" chemistry) has been used extensively to functionalize surfaces with macromolecules in a straightforward manner. We have previously developed a procedure using the copper(I)-catalyzed click reaction to tether synthetic α-helical peptides carrying two alkyne groups to a well-ordered azide-terminated alkanethiol self-assembled monolayer (SAM) on a Au(111) surface. While convenient, click-based strategies potentially pose significant problems from reagents, solvents, and reaction temperatures that may irreversibly damage some molecules or substrates. Tuning click chemistry conditions would allow individual optimization of reaction conditions for a wide variety of biomolecules and substrate materials. Here, we explore the utility of simultaneous SAM formation and peptide-attachment chemistry in a one-pot reaction. We demonstrate that a formerly multistep reaction can be successfully carried out concurrently by mixing azide-terminated alkanethiols, CuCl, and a propargylglycine-containing peptide over a bare gold surface in ethanol and reacting at 70 °C. X-ray photoelectron spectroscopy (XPS), surface infrared spectroscopy, surface circular dichroic (CD) spectroscopy, and scanning tunneling microscopy (STM) were used to determine that this one-pot reaction strategy resulted in a high density of surface-bound α-helices without aggregation. This work demonstrates the simplicity and versatility of a SAM-plus-click chemistry strategy for functionalizing Au surfaces with structured biomolecules.

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1. Introduction

Mediating the interactions between biological materials and inorganic surfaces is crucial for successfully integrating proteins and other biomolecules into abiological environments. However, controlling the interactions at this bio/abio interface is fundamentally difficult because the chemical, structural, and electrostatic environment that occurs near and on inorganic surfaces is significantly different from dilute aqueous buffers in which biomolecules evolved and are generally studied. To this end, a number of approaches have been used to immobilize biomolecules such as proteins on artificial supports. Immobilization techniques often use tailored non-covalent interactions with an artificial support or covalent interactions, which tether the biomolecule to the artificial support [1–4]. Many of these methods are straightforward and can lead to stable architectures. However, some limitations to known methods include loss of the biomolecule over time, the requirement of difficult or complex modifications to the biomolecules, or undesired and uncontrolled interactions with other molecules.

Our laboratory has generated surfaces composed of a dense monolayer of α-helical peptides tethered to a gold substrate and oriented with the helical backbone parallel to the substrate. This was accomplished through a two-step preparation procedure: 1) formation of an azide-terminated self-assembled monolayer (SAM) of alkanethiols on a Au(111) surface; followed by 2) a Cu(I)-catalyzed Huisgen cycloaddition ("click" chemistry) of two alkyne-containing unnatural amino acid residues in the peptide sequence with the surface-bound azide [5–8]. X-ray photoelectron spectroscopy (XPS), vibrational spectroscopy, circular dichroic (CD) spectroscopy, and scanning tunneling microscopy (STM) have been employed to fully characterize the chemical composition, topography, structure, and reactivity of these surfaces. In these studies, as well as the current work, we employed a synthetic peptide of sequence LKKLXKLLKXKLXKLK X, where X is the artificial amino acid propargylglycine, which acts as the reagent in the click reaction. (Hereafter we refer to the peptide as α11KL(CH3).) The pattern of alternating leucine and lysine residues induces an α-helical secondary structure by generating hydrophobic (leucine) and hydrophilic (lysine) faces on opposite sides of the helical rota-
tional plane. The hydrophobic proparglyglycine side chains are then immersed in the leucine environment by a distance that can be varied by changing the location of the proparglyglycine in the sequence of the peptide [9]. This helical structure is preserved when the peptide is bound to a SAM surface [6–8]. Fig. 1 shows an energy minimized structure of α11KL(CH) calculated with Avogadro, an open-source molecular builder and visualization tool, using the MMFF94 force field [10]. Our previous two-step optimized reaction procedure involved extensive handling, including substrate manipulation, exposure to heat and reactive reagents, rinsing with multiple solvents, surface characterization, and exposure to ambient conditions, all of which may irreversibly damage or destroy the surface, the monolayer, and/or the biomolecule.

Our interest in investigating an integrated approach that minimizes sample handling and damage caused by multistep procedures is prompted by the widespread observation that alkanethiol-based SAMs tend to degrade when exposed to even moderately harmful chemical or physical environments. Although alkanethiol-based SAMs are used as model surfaces for click reactions because they are easy to prepare and functionalize [11], they are fragile and susceptible to damage in multiple ways. The primary modes of damage originate from the oxidation of the sulfur head group and the exchange processes that are facilitated when a SAM is immersed in solution. For example, exposure to high temperatures in ambient conditions can lead to desorption or other decomposition of the SAM through the oxidation of the sulfur head group into sulfoxides or sulfonates, then desorb into solution [12,13]. Immersing an alkanethiol SAM to any solvent promotes thiol exchange, particularly at monolayer and substrate defect sites [13–17]. Additionally, the sulfur head group is prone to oxidation forming sulfone, sulfinyl, or sulffide groups when exposed to UV radiation, oxygen-containing solutions, or even air in complete darkness [18–21]. Oxidized thiols are less likely to be removed from the surface in air, but are solvated and desorb once they are immersed in a solvent. Because the prime starting points for exchange and other deleterious processes are in disordered and defect-filled areas, a higher quality SAM will slow the initiation and progression of these processes [13,22,23]. Unfortunately, defects are impossible to eliminate completely, and so subsequent chemical and physical exposure should be designed to minimize SAM damage to the greatest extent possible.

To address these known problems, here we explore a strategy in which the initial SAM formation step is integrated with the click chemistry into a single procedure, thereby minimizing substrate handling and subsequent damage to the surface. By combining the two sequential steps into a single one-pot reaction, we demonstrate additional versatility and control for generating peptide-terminated surfaces that takes advantage of the benefits of click chemistry [24]. This method allows for a trade off between solvent, catalyst concentrations, temperature, and reaction times, which accounts for the experimental requirements of a large variety of substrates, surface chemistries, and biomolecules.

We report the investigation of biofunctionalized surfaces through X-ray photoelectron spectroscopy (XPS), surface infrared spectroscopy collected in a grazing angle configuration, surface circular dichroic (CD) spectroscopy, and scanning tunneling microscopy (STM), in order to fully characterize the extent of reaction, and the structure and density of the resulting peptide termination.

2. Materials and methods

2.1. Substrate preparation

The procedure used to prepare the substrates in these studies has been described in detail in Ref [6]. Substrates used in this study were: Si(111) wafers (NOVA Electronic Materials) coated in 10 nm chromium and 100 nm gold for XPS and IR studies; fused quartz microscope slides (Quartz Scientific, Inc.) coated with 3 nm of chromium and 10 nm of gold for CD measurements; and mica substrates (Agilent Technologies) coated with 150 nm gold for STM imaging, purchased from Agilent Technologies. All chemicals were obtained from Sigma-Aldrich and used as received unless otherwise stated. Prior to the surface functionalization procedure, gold-on-silicon substrates were immersed for 1 min in a piranha solution (3:1 concentrated sulfuric acid: 30% hydrogen peroxide, caution: highly reactive, is explosive in the presence of organic contaminants), rinsed with high purity water (HPW, > 18 MΩ cm), concentrated hydrochloric acid, and ethanol, and dried with a nitrogen stream. Substrates were annealed with a hydrogen flame for 5 min and allowed to cool completely before use. Quartz substrates were only rinsed with ethanol and dried with a nitrogen stream before their use. Gold-on-mica substrates went through the same hydrogen annealing process as the gold-on-silicon substrates but were not rinsed in any solutions prior to their use.

2.2. Concurrent SAM formation and click reaction

A gold substrate was immersed into a 20 ml scintillation vial containing a well-mixed 4 ml ethanol solution with 0.25 mM 11-azide-1-undecanethiol (AzUDT), 10 μM tris(1-benzyl-1H-1,2,3-triazol-4-yl)methylamine (TBTA), 2 or 5 μM of α11KL(CH) (WuXi AppTec), and 0–100 μM of CuCl. A control sample was prepared using the same reagents AzUDT, TBTA, α11KL(CH), excluding the CuCl. A separate control was prepared including CuCl but using 1-decanethiol instead of AzUDT. TBTA and CuCl were first dissolved in acetonitrile to ensure they would fully dissolve in ethanol. The sealed reaction vial was heated at 70 °C for 4 or 24 h and afterward, the sample was allowed to cool undisturbed on the laboratory bench while still immersed in the solution. The sample was rinsed with ethanol, HPW, phosphate-buffered saline (PBS), HPW, and ethanol and then dried with a nitrogen stream. Functionalized gold-on-mica samples were rinsed only with ethanol and then dried with a nitrogen stream.

2.3. Surface characterization

Grazing incidence angle reflection-absorption infrared spectroscopy (GRAS-IR) was performed using a Bruker Vertex 70 Fourier transform infrared (FTIR) spectrometer equipped with a A518/Q horizontal reflection stage that illuminated the sample with p-polarized light at a grazing angle of 80° with respect to the surface normal, as has been described extensively before [6]. All spectra were normalized to a common CH₂ asymmetric stretching band at 2918 cm⁻¹. The instrument’s OPUS software was used to integrate the area beneath the azide adsorption peak at 2101 cm⁻¹ and
the amide I and II bands at 1500–1700 cm\(^{-1}\) in normalized spectra to quantify the extent of reaction. Experimental errors were calculated using the standard deviation of at least 3 measurements.

A Kratos Azis Ultra XPS with a monochromatic Al K\(x\) X-ray source was used to characterize the chemical composition of the functionalized surfaces, as has been described before [6,7]. A Jasco J-815 CD spectrometer was used to characterize the secondary structure of the bound peptide on a gold-on-quartz surface as has been described before [7]. An RHK Technology ATM 300 STM was used to obtain high-resolution images of the functionalized surfaces at ambient temperature and pressure. Samples were imaged using a 0.3–0.5 V bias and a 10 pA tunneling current. STM tips consisted of mechanically cut platinum/iridium wire (80% Pt/20% Ir, Nanoscience). Image processing consisted of using a masked high-pass filter fitting procedure to flatten the image, correct the plane tilt, and remove noise along the fast-scan direction due to low-frequency vibrations [25].

3. Results

3.1. X-ray photoelectron spectroscopy

XPS is a powerful tool for determining chemical composition and functional groups in a highly surface-specific manner. XPS was performed on peptide-terminated surfaces produced through the one-pot reaction integrating SAM formation with click chemistry. This analysis was used to confirm that the peptide was in fact reacting with the concurrently formed AzUDT SAM. Fig. 2 shows XPS spectra for the C 1s region near 285 eV for a peptide-terminated surface prepared from a solution of 5 μM peptide/25 μM CuCl (top) and a control surface prepared from a solution containing 5 μM peptide/0 μM CuCl (bottom) reacted for 4 h. All surfaces were prepared in solutions containing 0.25 mM AzUDT and 10 μM TBTFA. The XPS spectra corresponding to these samples were corrected to account for sample charging; observed binding energies were adjusted by between 0.6 and 1.0 eV for various samples to place the maximum intensity of the peak attributed to C–C at 285 eV and the lowest energy peak of both N 1s spectra at 401 eV [26–28]. This procedure adjusts the absolute magnitude of the observed core orbital energies, but does not change the relative difference in energy from perturbations to those orbitals. In both spectra, a strong C–C peak at ~285 eV (blue) and a second peak from C bonded to the more electronegative N atom in the amide backbone as well as residual azide (\textit{vida infra}) at 286 eV (red) are clearly visible. However, it is only in the sample exposed to 25 μM catalyst (top) in which peptide is abundant enough on the surface for a third peak corresponding to C=O from the peptide backbone to become visible at 288 eV (green). The control sample (bottom) does not contain any catalyst and the small amount of peptide bound to the surface does not yield a significant peak. While it is not entirely surprising that the reaction proceeded to a small extent even without the CuCl catalyst at the 70 °C reaction temperature (discussed further below), results from Fig. 2 shows that the higher catalyst concentration generated a surface with significantly more surface-bound peptide than when the catalyst is not used. We therefore refer to these surfaces as “reacted” and “unreacted,” respectively.

An alternative XPS parameter that could potentially be useful for surface coverage quantization is the N 1s peak at ~400 eV. N 1s signals from azide N atoms should be distinguishable from N atoms in a peptide backbone or terminal NH\(_2\) group. Representative XPS spectra for N 1s region for samples generated from 5 μM peptide/25 μM CuCl (top) and 5 μM peptide/0 μM CuCl (bottom) reacted for 4 h (previously described as “reacted” and “unreacted”) are shown in Fig. S1 (Supporting information). The reacted sample (black) showed the characteristic azide’s two-peak signature at 401 and 405 eV for the azide nitrogen atoms. The reacted sample (red) displayed a single peak representing the nitrogen atoms from the peptide backbone. At first glance it would appear that the characteristic azide peak at 405 eV has disappeared because of extensive reaction with the surface-bound azide, resulting in a strong and relatively sharp N 1s peak at 401 eV, corresponding to N atoms in the amide backbone. However, infrared spectra discussed below clearly showed that significant quantities of azide remained on the surface after the click reaction, and that this is actually to be expected. While measuring N 1s signals, we observed changes in the XPS spectra of the N 1s region near 400 eV with each consecutive measurement, which strongly suggested that the monolayer was being damaged from the XPS measurement itself. For example, Fig. S2 (Supporting information) shows three measurements taken sequentially on the same area on a 100 % N\(_3\)–terminated surface. The left panel shows the characteristic doublet at 401 and 405 eV from the electron-rich and electron-poor azide nitrogen atoms, as well as a third peak at ~399 eV observed in previous XPS N 1s spectra of azide-terminated SAMs [6]. Long acquisition times were necessary to accumulate high-resolution spectra, but with each measurement that follows (middle and right panels), the peak near 399 eV continued to grow. On the second and third runs, the low-energy azide nitrogen peak decreased and the high-energy azide nitrogen peak is difficult to distinguish from the noise in the spectrum. Careful inspection of the “unreacted” surface in Fig. S1 shows the growth of a shoulder at ~399 eV, which appears to be damage to the SAM layer after only a single scan. These spectra demonstrate sample degradation by XPS measurements even under UVH and in the absence of contaminating O\(_2\). Because of this, we suggest that any XPS data of azide functional groups should be only qualitative, and should be interpreted with the possibility of damage in mind.

3.2. Infrared spectroscopy

Surface infrared spectroscopy is an alternative characterization method that provides chemical and structural information with significantly less possibility for damaging the sample \textit{in situ}. Fig. 3 shows GRAS-IR spectra for the concurrently formed peptide-
reactive functional groups on the peptide are \( \sim 20 \text{ Å} \) apart, and the dimensions of the peptide obstruct attachment with azides directly beneath and along the edges of the bound peptide. Therefore, it is not surprising to find unreacted azide remaining on the SAM surface; the thiol component provides a substantially larger number of azide groups then required for the peptide reaction in the fully assembled SAM.

The control sample containing AzUDT, peptide and TBTA but excluding the CuCl catalyst (Fig. 3, black), shows similar peaks to the reacted sample (red) but in different proportions. Significantly more azide remained on the AzUDT control surface as shown by the larger peak at 2101 cm\(^{-1}\). Weak absorptions at 1662 and 1539 cm\(^{-1}\) corresponding to the peptide backbone indicate that a small amount of peptide is bound to the surface. Indeed, the uncatalyzed Huisgen cycloaddition has been shown to proceed at high temperatures but requires longer reaction times [30,31].

When combined with the XPS results of the catalyst-free control reaction (Fig. 2), these results demonstrate that a small amount of peptide can react with the surface at high temperature in the absence of CuCl. However, incorporating the CuCl catalyst permits the reaction to proceed to completion in only 4 h. We did not investigate how long it would take to generate a fully peptide-terminated surface in a catalyst-free reaction or if the SAM would remain intact under those high temperature conditions, but as we discuss below, this observation may allow samples that cannot be exposed to the Cu(I) catalyst to still employ Huisgen cycloaddition chemistry successfully.

The azide and amide regions of the vibrational spectra provide two convenient and complementary spectroscopic markers for tracking the progression of surface functionalization: the integrated area of the azide peak is inversely related to the extent of reaction between a formed SAM and the peptide, and the integrated areas of the amide I and II regions are directly related to the amount of the peptide on the surface after reaction and rinsing. These results from normalized GRAS-IR spectra of samples reacted with 2 and 5 \( \mu \)M peptide concentrations at various Cu catalyst concentrations for 4 and 24 h are shown in Fig. 4. Fig. 4A depicts the integrated area under the azide peak at \( \sim 2101 \text{ cm}^{-1} \) for two concentrations of peptide (2 \( \mu \)M, top, and 5 \( \mu \)M, bottom) and for two reaction times (4 h, black, and 24 h, gray). Similarly, the integrated area for the amide I and II absorption bands are shown in Fig. 4B for the two concentrations and reaction times. The measured amount of azide on the surface should be highest for the most extensive reaction, while the measured amount of amide absorption on the surface should be highest for the most extensive reaction. Indeed, the trends between the two absorption bands are clearly inversely proportional when comparing Fig. 4A and B. All four panels of Fig. 4 show that the greatest extent of reaction occurred at a concentration of CuCl of 25 \( \mu \)M for all solution reaction conditions. When comparing the difference in concentration of peptide used in the reaction (2 or 5 \( \mu \)M, top and bottom panels, respectively) at this catalyst concentration, the 5 \( \mu \)M reactions always went to greater extent (smallest azide band, largest amide bands) than the corresponding reaction with 2 \( \mu \)M of the \( \alpha \)-helical peptide. Finally, when comparing reaction times (4 or 24 h, black and gray bars, respectively), the 24 h reaction time always produced a greater extent of surface peptide functionalization than the 4 h reaction time. We observed that with 5 \( \mu \)M peptide, the reaction proceeded with the smallest amount of catalyst when maintained for 24 h. As mentioned before, this is not unexpected because even the uncatalyzed click reaction control (Fig. 3, blue) showed that the reaction proceeded to a small extent in 4 h. Furthermore, when the solution contained at least 5 \( \mu \)M of peptide and 25 \( \mu \)M of CuCl, we observed that the surface was almost fully reacted by the end of 4 h and that the decrease in the amount of azide and the increase
of amide I and II was not significant between the 4 and 24 h reactions. Taken together, this evidence demonstrates that peptide and catalyst concentration and reaction time can be balanced if necessary for particularly sensitive materials or substrates that will not endure extensive reaction times, harsh chemical conditions, or exposure to high concentrations of the copper catalyst.

3.2. Circular dichroic spectroscopy

The high temperatures and organic solvent used in these studies could potentially be problematic for maintaining the structure and function of most biomolecules. The secondary structure of the surface-bound helix generated from 5 μM α11KL(CH) and 15 μM CuCl reacted for 4 h was checked by surface CD spectroscopy as has been described before [7], and showed strong absorptions at ∼208 and 219 nm, indicating a well-structured α-helix (Fig. 5).

3.3. Scanning tunneling microscopy

The surface-bound helix produced through the one-pot reaction was imaged by STM at low current to characterize the topography and peptide density of a fully reacted surface, compared to a control sample composed of a decanethiol SAM exposed to 5 μM α11KL(CH), TBTAC, and 100 μM CuCl (the maximum amount of catalyst used in these studies). The high-resolution capabilities of STM ensure that even moderate deformation of the SAM would be apparent in images. Fig. 6 shows STM images of the decanethiol

Fig. 4. Integrated area beneath the azide (A) and amide I and II regions (B) of normalized spectra of peptide-functionalized surfaces. Reactions occur with 2 or 5 μM α11KL(CH), top and bottom graphs respectively, while the CuCl concentration is varied from 1 to 100 μM for 4 h (black bars) or 24 h (gray bars). Error bars are the standard deviation of at least three measurements.

Fig. 5. Circular dichroic spectra of a peptide-terminated surface formed through concurrent SAM and click reactions. Troughs near 208 and 219 nm indicate the peptides have strong α-helical character.
control (A) and the reacted surface (B), with scale bars of 10 nm on both images. On the control substrate (A) we observed a smooth surface with sharp edges. The surface contained a small number of bright features, some of which are circled in Fig. 6A, that were measured to be 2 \times 3 \text{ nm} in size. These dimensions correspond to the width and length of the energy-minimized α-helical peptides used in these experiments, which have been observed in previous STM studies in our laboratory of surface-bound αI1KL(CH) [8]. We therefore assigned these features as physisorbed peptides on the decanethiol SAM surface. To calculate the surface coverage of these unbound peptides, we determined the fraction of pixels assigned as peptide features from the total number of pixels of the entire image, and determined that only 5% of the surface of control samples were covered with peptides. This result may seem in contrast with the results of a similarly prepared control sample examined by GRAS-IR (Fig. 3, blue), in which no peptide amide modes were detected on the control surface. Although GRAS-IR is optimized for sensitivity of surface-associated absorptions, 5% of a monolayer is an extremely small amount of material for a surface-averaged measurement technique, and we would not expect to see signal from such a small amount of material in our current instrument configuration. This result from STM therefore allows us to detect physisorbed peptides below the resolution of our GRAS-IR experiment, and emphasizes the importance of both surface-averaged and molecular resolution characterization methods in studying single-monomer biomolecular surfaces.

Although the control sample would ideally display no physisorbed peptide at all, the presence of a small amount of peptide on this surface was not surprising since the samples created for STM characterization were only rinsed with ethanol. Rinsing with PBS, as was done for samples applied to all other surface characterization techniques discussed here, has been shown to reduce the amount of physisorbed proteins on a surface [32,33], possibly because ions in the buffer compete with the peptide, particularly with the charged lysine side chains, for adsorption on the surface [34]. Samples prepared for STM imaging that did include PBS rinsing were difficult to optimize as they demonstrated a significant amount of tip-sample interactions, attributed to residual buffer salts on the sample. While rinsing with PBS buffer has been incorporated into this set of studies, we have previously shown through vibrational spectroscopy and STM characterization that the prior two-step functionalization procedures produced surfaces with very little physisorbed peptide. Here we include PBS rinsing as an additional aid to remove physisorbed peptide.

In Fig. 6B, the image of the fully reacted surface showed step edges that were less distinct from the control sample, as well as raised features corresponding to peptide functionalization. Identifying individual peptides bound to the surface is not as straightforward to do by eye, but the black box on Fig. 6A marks an area where the structure of bound peptides are relatively clear. However, image analysis of these peptides clearly revealed a pattern of regularly spaced features 2 \times 3 \text{ nm} in dimension covering a significant portion of the surface. Image analysis conducted by calculating the fraction of pixels assigned to peptides from the total number of pixels from the entire image resulted in ~78% of the surface covered by these peptide features. This is essentially identical to surface coverage results from previous studies which employed the sequential, two-step procedure [6]. Although we cannot analyze the SAM itself to compare with the surface created through the two-step procedure, the observation that the end product is the same is the strong evidence that the SAM layers are highly similar in both cases. On this surface, we also found occasional bright features smaller than the 2 \times 3 \text{ nm} features, with heights that placed them above the surrounding peptide monolayer. We attribute these features, one of which is shown circled by a black ring in Fig. 6B) to α-helical peptides physisorbed on the surface and bound to the SAM surface by only one of the two available propargylamine residues in the sequence [8]. In this case, a peptide would physically protrude and appear brighter than the surrounding monolayer, but lack the induced helical structure of the energy minimized helix stapled at two points to the SAM surface. We have previously observed that even a highly-optimized sequential surface preparation method, SAM formation followed by click chemistry, results in only approximately two thirds of the peptides at the surface attached to the surface through two bonds [2]. It is therefore not surprising that this one-pot simultaneous preparation reaction leads to some small fraction of the surface terminated in peptides attached at only one propargylamine.

4. Discussion

This investigation was prompted by the concern that the goal of preparing biomolecule-based surfaces and materials is often hampered by the fact that solution-based chemistries could be harmful to the molecules and materials intended for application.
both to the biomolecule, in this case the peptide, and to the surface to which it is reacted, in this case SAMs on Au(111). For example, the presence of click reaction reagents such as TBT, CuCl, and α11KL(CH) could interfere with and disrupt the complex and dynamic alkanethiol monolayer as the weakly bound alkanethiols desorb and readsoorb [12,14,15,17]. The click reaction reagents could also interact with the gold surface and potentially compete with the alkanethiols in solution for available adsorption sites. A more complicated scenario occurs when the click reagents also react with the alkanethiols, preventing their adsorption or contributing to further desorption on the surface. Furthermore, the sulfur head group of the alkanethiol is susceptible to oxidation reactions that create species that are easily desorbed into solution, damaging the SAM. Initially, we were simply interested in addressing these concerns by adding excess AzUDT to the click reaction solution over a preformed SAM. This excess AzUDT would backfill exposed adsorption sites during the reaction, providing a mechanism for SAM healing during the potentially harsh click reaction. The success of this preliminary work led us to try to carry out both self-assembly and click reactions simultaneously so the whole peptide-terminated surface would self-assemble along with the alkanethiol monolayer.

Monolayer formation in solution complicates the thermodynamics and kinetics of the self-assembly processes of alkanethiols on the Au(111) surface because of the competing solvent-Au interactions, solubility, and stabilizing solvent-alkanethiol interactions on the surface. Thus, alkanethiol monolayers can be formed to varying levels of order, controlled to some extent by the solvent characteristics. Nonpolar organic solutions have improved thiol-solvent interactions but produce surfaces with less order. Alkanethiols are less soluble in polar solvents, however higher quality surfaces are produced. The use of an aqueous solution would be more amenable to preserving biomolecule structure and function, however we decided to use ethanol as the solvent in these studies as it is the most commonly used solvent to form alkanethiol SAMs. Ethanol is also well known to produce ordered surfaces with fewer defects, and we have confirmed using CD of the peptide-terminated surface that the helicity of α11KL(CH) is preserved on the surface by the position of the propargylgycine side chains along the helical structure. It is possible that other biomolecules could be altered from exposure to the ethanol solvent, and investigators attempting to use this one-pot procedure will have to consider and test solvent systems carefully.

The copper sulfate/sodium ascorbate system has been widely used as the method for generating Cu(1) in situ, where it is needed to catalyze the click reaction. This development was crucial in making click reactions possible in aqueous environments and thus for allowing the attachment of a wide variety of biological molecules [35]. In these studies, we use a Cu(1) source directly to negate the need of the sodium ascorbate reducing agent [36–38]. Initial studies in which we tried to incorporate the sodium ascorbate demonstrated that its presence was detrimental to the formation of the alkanethiol monolayer and led to a large amount of physiosorbed peptide. In fact, we observed that an alkanethiol monolayer did not appear to form at all and instead peptide aggregated onto the gold surface. Under our reaction conditions, CuCl can oxidize [26] and we did not have an active reducing agent in solution. We thus conducted these reactions under significantly higher concentrations of the copper catalyst than is commonly used for click chemistry, up to 100 μM, to react the surface completely. The incorporation of CuCl into the reaction also required the use of a co-solvent of acetonitrile to dissolve it, as it is not soluble in ethanol solutions. GRAS-IR spectroscopy of the SAM surface demonstrated that the acetonitrile did not appear to compromise the quality of the monolayer, but again, is a condition that will have to be considered for each surface being fabricated.

Because we are coordinating the formation of the alkanethiol self-assembled monolayer with the click reaction tethering peptides to the forming or formed surface, we compared the quality of the peptide-functionalized surface with that of the surface generated through a previously reported two-step sequential procedure (SAM formation then peptide functionalization). For all the reasons discussed above, the addition of the click reaction reagents to the azide-terminated alkanethiols could potentially significantly complicate monolayer formation, as alkanethiols in solution compete with solvent, CuCl, TBT, peptide, and reacted species for adsorption sites. This could potentially reduce the order of the monolayer structure, particularly when compared with surfaces in which a stable and ordered monolayer was fabricated first, before peptide termination. However, STM images of the decanethiol control surface formed with a solution containing all of the click reagents, including the CuCl at the highest concentration, confirm the formation of an ordered surface despite the presence of these additional species (Fig. 6A). Thiols have a thermodynamic and kinetic advantage to remaining on the surface through the formation of the Au-S bond, minimizing the surface energy, and the large excess of AzUDT in solution. This does not take into account the presence of reacted peptide-thiol complexes in solution which would also compete for surface adsorption and which would eventually need to be expelled from the forming monolayer on the surface. In the end, our two-step procedure yields higher quality surfaces than the concurrent reaction, but this may not be the case for other reaction mixtures in which the reaction solution does irrepairable damage to the SAM surface and a concurrent reaction produces a somewhat defective, but higher quality surface. The combined one-pot reaction provides this option.

In the concurrent one-pot reaction, the concentration of the azide-terminated thiol is always significantly greater than any other reaction component at the equilibrium conditions, dictating that the AzUDT has a significantly higher probability of encountering the surface first to begin building the SAM onto which the peptides will eventually be tethered. SAM formation is generally known to occur though two steps: 1) initial adsorption and formation of the sulfur-gold bond, which takes place within minutes of exposure; followed by 2) an ordering process in which the alkane chains stand up from the gold surface to maximize the number of molecules attached on the surface and van der Waals interactions between molecules [12,14,15,17]. This second step is known to take much longer than the first, and occurs on the order of hours. At room temperature, SAMs are typically left in the corresponding solution for ~24 h to ensure a well-ordered surface. In order to reduce this SAM formation time, we conducted our reactions at 70°C to take advantage of higher deposition temperatures that encourage desorption of weakly bound thiols and promote the formation of well-ordered monolayers in a shorter time [27,39]. High temperatures could also effect the secondary structure of the peptide, but we have previously shown [7] that while the α11KL(CH) peptide loses some helicity at high temperatures it regains helical character upon cooling. Indeed, the peptide’s helical character is reinforced once the peptide is tethered to the alkanethiol surface in order to place the reactive propargylgycine residues in an appropriate orientation to maximize the Huisgen cycloaddition, confirmed by the preservation of the helicity of the α11KL(CH) peptide bound on the surface through concurrent reaction measured by surface CD.

One of the biggest obstacles for the concurrent SAM formation and peptide-tethering reaction to occur is the attachment of unbound azide-terminated thiols and peptides in solution; the click reaction will yield peptides with attached thiols at one or both reactive residues. The ordering of an alkanethiol monolayer relies on the removal of defects based on the reduced stability of thiols in these areas. We propose that reacted complexes are less likely to adsorb on the surface based on their limited numbers in solu-
tion, and because they are much larger and bulkier than unreacted thiols, they are still more likely to be desorbed from the surface as defects. The sporadic presence of bright, circular or ellipsoidal features throughout the STM image in Fig. 6B could indicate the existence of partially reacted complexes attached to the surface within the SAM. Because of this, the copper catalyst concentration is crucial in determining the optimal surface functionalization parameters; higher catalyst concentrations permit higher reactivity but could limit adsorption of the desired alkanethiol-peptide construct, while lower concentrations of the Cu(I) source would require longer reaction times. Similarly, the concentration of the peptide contributes to the extent of reaction and therefore, the amount of peptide that is subsequently attached onto the surface.

We observed that even with no Cu(I) catalyst the reaction is able to proceed, albeit slowly, and at the lowest concentrations of Cu(I) catalyst, the reaction progressed at long enough reaction times. We determined that the optimum conditions for combining the SAM formation with the peptide surface functionalization for a lower concentration (2 μM) of peptide is 25 μM CuCl at both long and short reaction times. However, at a higher peptide concentration (5 μM), the optimum conditions range between 10 and 25 μM CuCl for the 4 h reaction time. This range of possible reaction conditions allows this methodology to be applied to a variety of biomolecule and substrate systems, where competing deleterious effects of temperature, solvent, and exposure to chemicals can be balanced to achieve a particular outcome. For example, many biological molecules of interest will not remain stable for extended periods of time at 70 °C. Increasing the concentration of the CuCl while decreasing the length of reaction time can compensate for this. Alternatively, applications of this surface chemistry for generating electronically active surfaces might not withstand the presence of inevitable copper contamination from the Cu(I) catalyst, but this can be eliminated from the reaction solution by increasing reaction time and temperature.

5. Conclusion

In conclusion, this work demonstrates the successful integration of click chemistry with a SAM-forming reaction to generate peptide-functionalized SAM surfaces on Au. Employing a Cu(I) source directly as the catalyst and a vast excess AzDUT permits the formation of an underlying SAM surface. The use of an organic solvent and high temperatures for the reaction aid in the formation of the SAM surface and allow the click reaction to proceed to completion in 4 h, based on GRAS-IR data. CD of the final surface demonstrates that the peptide maintains a helical structure when bound to the surface through these reaction conditions. As the catalyst and peptide concentration, temperature, and reaction time are coupled, these reaction parameters can be used to optimize the procedure and could be tailored to achieve a surface structure with the desired extent of reaction, coverage, and quality, enlarging the SAM-plus-click reaction toolkit for biomolecule-based surface functionalization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsusc.2016.10.036.

References


