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Photoactivated Bioconjugation Between *ortho*-Azidophenols and Anilines: A Facile Approach to Biomolecular Photopatterning

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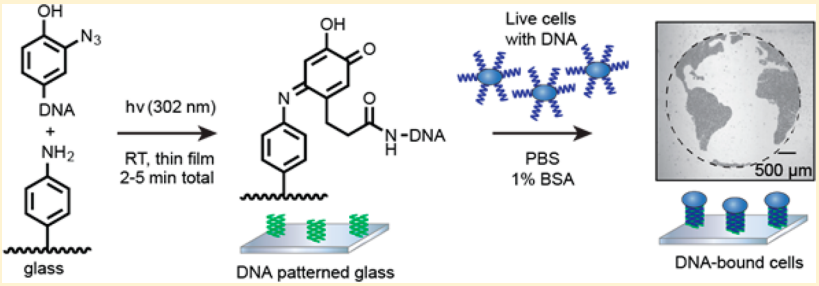
Supporting Information



ABSTRACT: Methods for the surface patterning of small molecules and biomolecules can yield useful platforms for drug screening, synthetic biology applications, diagnostics, and the immobilization of live cells. However, new techniques are needed to achieve the ease, feature sizes, reliability, and patterning speed necessary for widespread adoption. Herein, we report an easily accessible and operationally simple photoinitiated reaction that can achieve patterned bioconjugation in a highly chemoselective manner. The reaction involves the photolysis of 2-azidophenols to generate iminoquinone intermediates that couple rapidly to aniline groups. We demonstrate the broad functional group compatibility of this reaction for the modification of proteins, polymers, oligonucleotides, peptides, and small molecules. As a specific application, the reaction was adapted for the photolithographic patterning of azidophenol DNA on aniline glass substrates. The presence of the DNA was confirmed by the ability of the surface to capture living cells bearing the sequence complement on their cell walls or cytoplasmic membranes. Compared to other light-based DNA patterning methods, this reaction offers higher speed and does not require the use of a photoresist or other blocking material.

INTRODUCTION

The ability to attach living cells to surfaces has enabled the study of many key behaviors in biology, including immune synapse formation,[1] stem cell differentiation,[2,3] cancer cell motility,[4] and drug response.[5] In most of these studies, cells of interest are exposed to surfaces, such as slides or supported lipid bilayers,[6−8] that have been patterned with biomolecules that engage cellular receptors in a well-defined way. Outside these experiments, immobilized biomolecule arrays have also shown promise in fundamental studies of biofuel production,[9−12] the investigation of antibody−antigen interactions,[13] and the construction of biofuel cells based on enzymes.[14] Although most of these studies have capitalized on interactions between the surface integrins of adherent mammalian cells and proteins bearing "RGD" peptide motifs,[15−18] our lab has developed an alternative strategy in which synthetic DNA strands introduced on the cell surfaces bind to sequence complements displayed on the binding surface.[19−26] While it does require the additional step of covalently modifying the cells, demonstrated advantages of this approach include its generality for all biological cell types, exceptionally high capture efficiency, and ability to generate complex multicellular patterns through the use of multiple DNA sequences.[22,23] In addition, the DNA-based adhesion event has been shown to exhibit minimal changes in cellular behavior because it does not involve native cell receptors.[24] In previous studies, this strategy was used to measure the metabolism of single cells,[21] conduct single-cell RT-PCR analysis,[26] study the diffusion of paracrine signaling molecules,[22] and connect cells directly to AFM tips.[20] The technique has also been applied to the formation of three-dimensional cell clusters in suspension.[27−29]

This cell capture method benefits greatly from the availability of streamlined techniques that can generate small and elaborate patterns of DNA with high precision and high throughput. This is typically done through the use of photolithography,[22,30−32] soft lithography,[33−35] Dip-Pen nanolithography,[36,37] inkjet printing,[38−40] and electron beam lithography.[41−43] Several of these methods have yielded impressive advances in the types of arrays that can be generated.[44−47] However, few if any can successfully combine the ability to generate submicron feature sizes with biomolecular compatibility, the use of ordinary

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laboratory equipment, and high patterning speed. This latter feature is particularly important for the future commercialization of these platforms for diagnostic use, for example.

The use of photoinitiated coupling reactions offers significant promise for surface patterning, as they can harness the inherent spatial precision and parallelization of photolithography without the need for photoresists. This feature serves to increase biocompatibility and obviate the need for multiple etching and washing steps. In this approach, the species of interest is first placed on a photoreactive surface, after which irradiation initiates the reaction to immobilize the molecule through a covalent linkage. This process has already been demonstrated with reactions that display low chemoselectivity, relying on highly reactive radical or nitrene intermediates generated from reagents like aryl azides[48,49] and benzophenones.[50] These intermediates insert into C−H, O−H, or N−H bonds found in the biomolecular targets. While these reactions have enabled the coupling of photoreactive biomolecules,[48−51] surfaces,[52] and caged species,[53] the low reaction specificity limits their applicability. Photoinitiated orthogonal bioconjugation reactions have also found use in this space, primarily with thiol−ene,[54,55] cyclopropenone-azide,[56] quinone methide,[57,58] and thioaldehyde[59,60] reactions. However, few of these reactions have been evaluated using thin films that are compatible with photomasking techniques, and many require additional steps for biomolecule attachment or precursors that are synthetically difficult to access.

As a new photolithographic strategy for the generation of complex patterns of DNA molecules on surfaces, we detail herein a new photochemical bioconjugation reaction and demonstrate its utility for immobilizing DNA-modified cells in desired patterns. The process is based on the photoinitiated reaction of aniline-modified glass surfaces with azidophenols, enabling the preparation of a monolayer of covalently attached single stranded DNA (ssDNA) on a surface upon UV irradiation. Feature sizes as small as 20 μm are demonstrated, and further improvements in resolution are likely possible. The subsequent introduction of living cells modified with complementary strands results in hybridization and immobilization, allowing the straightforward generation of complex cellular arrangements.

RESULTS AND DISCUSSION

The development of an effective, light triggered surface patterning strategy was predicated on our previous studies of a series of aniline-based oxidative coupling methods for site-specific bioconjugation.[61−65] These reactions proceed via the coupling of aminophenols and anilines (Figure 1a), presumably involving iminoquinone intermediate **2**. Although periodate ion can also be used as a terminal oxidant,[63] ferricyanide ion mediates particularly clean coupling to afford *p*-iminoquinone **5** as a single, stable product.[64] The reactions proceed very rapidly (typically <2 min using periodate and <15 min using ferricyanide) and can be used to modify biomolecules at low-micromolar concentrations. In previous work, these coupling strategies have been used to couple oligonucleotides,[66] polymers,[67] and chromophores[64] to aniline groups introduced on the surface of genome-free viral capsids and at the N-termini of antibody Fc domains.[68]

The success of these reactions is based on the generation of the key iminoquinone intermediate (**2**). While this is easily done through the oxidation of *o*-aminophenol precursors, a photochemical alternative could be envisioned by modifying

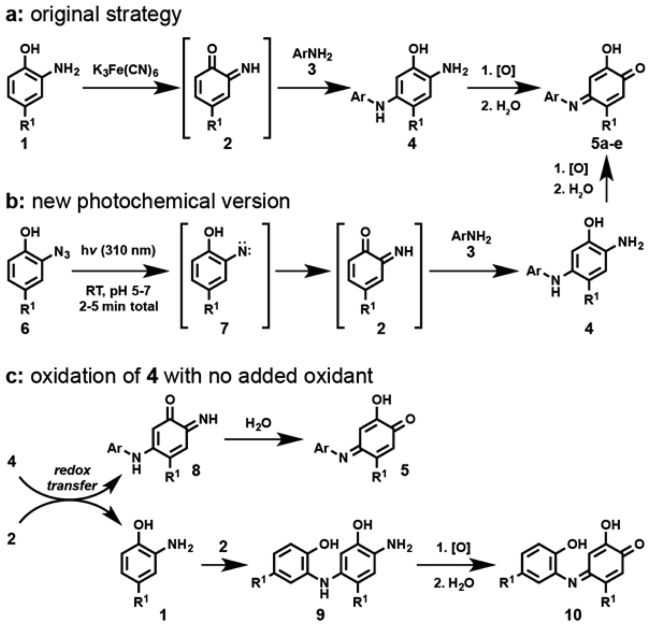

Figure 1. Rapid and efficient oxidative coupling reactions involving anilines and iminoquinone intermediates (**2**). (a) The previously reported reaction with aminophenol precursors requires ferricyanide as an oxidant, ultimately yielding product **5**. (b) The reaction of azidophenol **6** is initiated by UV light and requires no additional oxidant for the aniline coupling step to occur. This reaction presumably involves nitrene **7**, which rapidly rearranges to iminoquinone **2**. (c) In the absence of added oxidant, the oxidation of initial adduct **4** can occur with O_2 or through the redox transfer pathway shown.

the amine to include a leaving group that would be lost upon irradiation. Literature precedent suggested that, in fact, azidophenols had the potential to undergo such a transformation. In particular, early studies by Sander,[69] Koseki,[70] and Oleinik[71] demonstrated that irradiation of *o*-azidophenol (**6**, R^1 = H) in an argon matrix liberates nitrogen gas to give the corresponding nitrene (**7**). This intermediate rapidly rearranges to form iminoquinone **2**, providing a precedent that azidophenols could indeed serve as photolabile iminoquinone precursors. In other early work, Bayley has used a related pathway for the photogeneration of inhibitors of photosynthetic electron transport.[72] Our extensive studies involving 4-alkyl-substituted versions of the *o*-iminoquinone intermediates suggested that chemoselective bioconjugation to anilines would be possible once these groups had been generated, Figure 1b.

With this concept in mind, initial reaction studies involved the irradiation of 4-methyl-2-azidophenol (**6a**, $R^1 = CH_3$) in the presence of toluidine (**3**, Ar = 4-$CH_3C_6H_4$) as an aniline coupling partner at the absorbance maximum of **6a** (Supporting Information Figure S1). These reactants were exposed to 302 nm light for 5 min using a 6 W hand-held lamp in bis-tris buffer at pH 6. The sole toluidine-containing product was identified as **5a** ($R^1 = CH_3$), as characterized using reversed phase HPLC and NMR (Figure S2). The success of the reaction was found to be dependent on the ratio of the reacting species, with up to 95% HPLC yield being obtained with 1.5 mM **6a** and 100 μM **3** (Figure S2).

Although the reaction did not require an additional oxidant, the *p*-iminoquinone species was nonetheless obtained. This could occur from the aerobic oxidation of initial adduct **4** or

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from an intermolecular redox exchange with an additional iminoquinone molecule (**2**), Figure 1c. While the latter pathway yields a stable adduct involving the aniline group, it also produces an aminophenol byproduct that can couple competitively with an additional equivalent of **2** (albeit apparently more slowly). In support of this pathway, small amounts of dimeric species were isolated that were consistent in mass with compound **10**. The exact structure of this species is still under investigation, as only minimal amounts are available, and it has limited solution stability. In any case, this secondary reaction potentially limits the efficiency of this coupling strategy relative to our previous methods that involve an excess of additional oxidants. Additional experiments to date involving the addition of oxidants to the photochemical version of the coupling reaction have produced complex product mixtures, likely arising from the photochemical degradation of the oxidants themselves.

With the basic reactivity pattern in place, the biocompatibility of the reaction was next tested in the context of protein bioconjugation. The first of these experiments involved genome-free MS2 viral capsids (277 nM, 50 μM in capsid monomer) that possessed a *p*-aminophenylalanine (*p*AF) residue in position 19 of each monomer, Figure 2a.[62] This

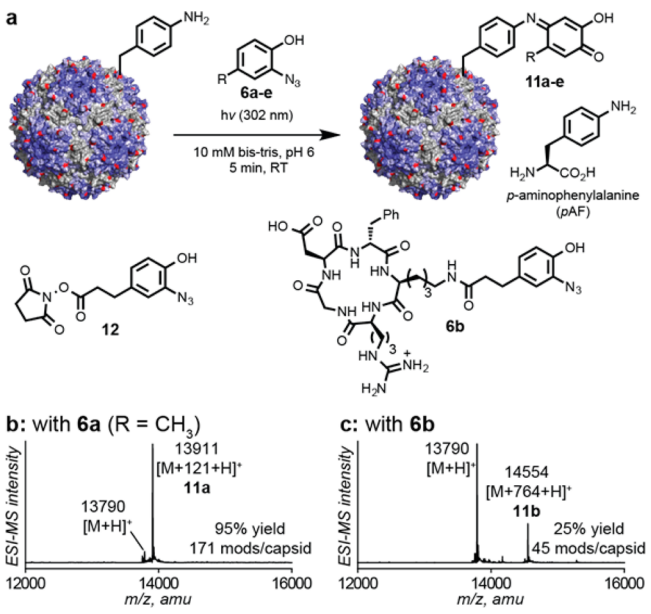

Figure 2. Modification of aniline-containing MS2 viral capsids with azidophenols under photochemical conditions. (a) Each of the 180 capsid proteins contained one aniline group in position 19. The intact structure (50 μM in aniline concentration) was irradiated in the presence of azidophenols. ESI-MS yielded the reconstructed mass spectra for reaction with (b) **6a** (R = CH_3) and (c) **6b**. A negative control irradiating **6a** with wild type T19 MS2 showed no reaction. Expected mass for the coupling product with **6a**: m/z = 13911; expected mass for the coupling product with **6b**: m/z = 14554.

residue was introduced using the amber codon suppression techniques developed by the Schultz lab,[73] providing 180 aniline groups on the external surface of each 27 nm protein shell. These capsids were combined with compound **6a** (R_1 = CH_3, 1 mM) in bis-tris buffer at pH 6. Following irradiation at 302 nm with a hand-held lamp for 5 min, excellent conversion was observed to the expected product, Figure 2b. In contrast, no coupling was observed in the absence of irradiation or for irradiated capsids that lacked the *p*AF residues (see Figure S3). The reaction reached complete conversion after 5 min of irradiation, with an optimal pH range of 5−7 (Figures S4−S6).

To access more complex substrates, *o*-azidophenol NHS ester **12** was prepared and coupled to a cyclic RGD peptide (yielding **6b**), PEG polymer chains (**6c**,**d**), and the 5′-terminus of a 20 nt DNA strand (**6e**, see Supporting Information for experimental details). All of these reagents could be prepared on the benchtop under normal conditions and were stable for months when stored at −20 °C in the dark. A 5 min exposure of *p*AF-containing MS2 capsids to **6b** (1 mM) in the presence of 302 nm light led to the formation of the expected product in about 25% yield, Figure 2c. The coupling of *p*AF-MS2 to PEG substrates **6c** and **6d** under similar conditions was monitored using SDS-PAGE, followed by staining with Coomassie blue (Figure 3a). Densitometry analysis indicated that 56% of the

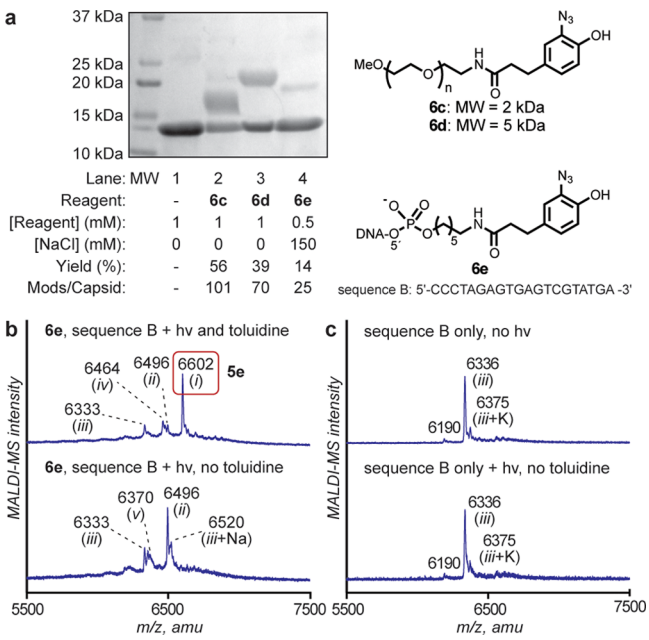

Figure 3. Modification of anilines with PEG polymers and DNA strands bearing 2-azidophenols. (a) *p*AF19 MS2 (50 μM in aniline groups) was buffered with pH 6 bis-tris (10 mM) and irradiated for 5 min with 302 nm light in the presence of **6c**−**6e**. (b) Oligonucleotide **6e** (sequence B, 500 μM) was irradiated at 302 nm for 5 min in the presence or absence of 2.5 mM toluidine. (c) In parallel, unmodified sequence B was subjected to the same irradiation conditions to confirm sequence integrity. All of the reactions were analyzed by MALDI-TOF MS. The peak assignments are (*i*) m/z = 6602: **5e**, the expected coupling product of **6e** with toluidine; (*ii*) m/z = 6496: **6e** after azide photolysis (loss of N_2); (*iii*) m/z = 6333−6336: amine-modified sequence B; (*iv*) m/z = 6464; and (*v*) 6370: abasic analogs of **5e** and photolyzed **6e** that are typically seen upon MADLI-TOF MS ionization.

monomers had been modified using the 2 kDa polymer and that 39% were modified using the 5 kDa polymer. These yields are lower than those achieved using aminophenols and external oxidants (perhaps due to the competing coupling pathway described above), but the results nonetheless confirm that useful levels of site-selective modification can be achieved under photochemical control. As a second substrate, an endocellulase from *Pyrococcus horikoshii* (EGPh) bearing a *p*-aminophenylalanine residue in position 2 was also modified with **6d** in 60% yield (Figure S7).

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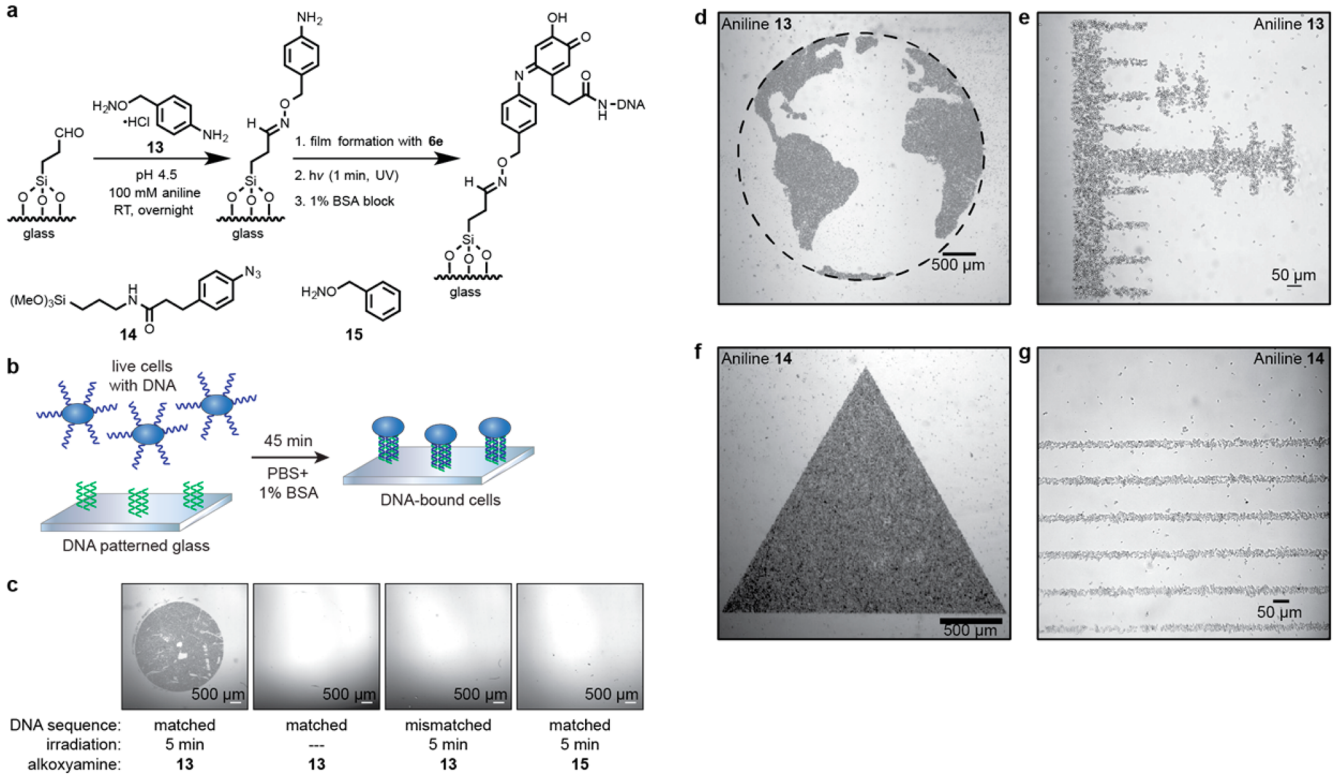

Figure 4. Cell immobilization via DNA hybridization on glass slides. (a) Aniline-coated glass surfaces were generated starting from aldehyde glass or through treatment with silane **14** followed by a TCEP wash. UV irradiation of the aniline surfaces coated with a thin film of azidophenol DNA yielded covalent modification of the glass surface. (b) After photopatterning, *S. cerevisiae* modified with a complementary DNA sequence was then exposed to the surface strands to allow hybridization to occur. (c) DNA-labeled *S. cerevisiae* was exposed to slide surfaces treated with (from left to right) (i) matched sequence **6e** and irradiation (positive experiment), (ii) matched sequence **6e** without irradiation, (iii) mismatched sequence **6e** and irradiation, and (iv) *O*-benzylhydroxylamine, followed by matched sequence **6e** and irradiation. (d–g) UV irradiation of dehydrated films of **6e** on aniline glass through a photomask, followed by addition of *S. cerevisiae* and gentle rinsing, resulted in the pattern shown (yeast appear as dark regions). Patterns were made of (d) the Earth with a dashed line indicating pattern edge, (e) a marker with 30 μm features, (f) a triangle with sharp vertices, and (g) a series of lines 20 μm in width. Aniline glass was generated from (d,e) alkoxyamine **13** or (f,g) silane **14**.

While the experiments above confirm that the reaction is compatible with peptides and proteins containing most amino acids, it was found to be incompatible with free cysteine sulfhydryl groups, as multiple addition products were observed. However, free cysteines can be capped with maleimides prior to irradiation, preventing further reactivity (Figure S8).

These protein-based experiments demonstrate that the aniline coupling reaction can be photoinitiated and still retain much of the high chemoselectivity, speed, and conversion levels that are characteristic of the parent strategy. To test its compatibility with the DNA strands required for surface patterning, 20 nt ssDNA **6e** was first coupled to toluidine. After 5 min of irradiation at 302 nm using a hand-held lamp, MALDI-TOF MS analysis indicated clean conversion to the expected product, Figure 3b (species *i*). In the absence of the aniline coupling partner, no reaction was observed other than the loss of nitrogen from the azide group (species *ii*). The parent DNA strand showed no change in mass upon irradiation under these conditions (Figure 3c). Strand **6e** was also coupled successfully to MS2 capsids containing *p*AF groups, as confirmed using an SDS-PAGE gel shift assay (Figure 3a, lane 4). Densitometry analysis indicated that about 14% of the monomers had participated in the reaction, corresponding to 25 DNA strands on each capsid.

Since DNA has successfully been used with primarily long-wave UV light in other applications,[30,32,74,75] our resulting bioconjugates were analyzed carefully as the exposure of oligonucleotides to UV light is known to result in thymine cyclobutane dimers and Dewar valence isomers.[76,77] Using a combination of dynamic light scattering measurements and SDS-PAGE gel shift assays, it was demonstrated that the DNA-MS2 construct was able to undergo successful hybridization after the photochemical bioconjugation step (Figure S10). To demonstrate that ability further, the melting temperature of two different ssDNA sequences and their complements was found to be unaltered by the irradiation step (Figures S11 and S12). While particularly problematic sequences, such as poly(T) motifs, may still be incompatible with this technique, these results suggest that a very large number of capture sequences can be used.

The adaptation of this reaction strategy for the covalent attachment of DNA to surfaces required the development and optimization of four independent steps: (1) the generation of an aniline-functionalized surface, (2) the deposition of a thin film of DNA conjugate **6e** (Figure S13), (3) the exposure of selected regions of the surface to light to initiate the reaction, and (4) the development of a rinsing protocol to remove unreacted DNA strands. The requisite aniline functionality was introduced onto glass surfaces using one of two methods. In the first, aldehyde-silanized glass was exposed to alkoxyamine reagent **13** in the presence of additional aniline[78,79] (Figure 4a). In the second approach, freshly plasma-cleaned glass slides were

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exposed to a solution of phenylazide silane **14** in acidic methanol (Figure S13a). In the latter case, the azide groups were subsequently reduced to anilines using TCEP before use. The final contact angles of the modified surfaces were estimated as 40° in both cases, suggesting the successful formation of the organic monolayer.

Following surface derivatization, a thin film of DNA was then deposited on the surface using a solution of **6e** in a 1:1 mixture of water and methanol (see Figure S14). The solution was distributed with a 1 cm-thick rectangular piece of polydimethylsiloxane as a spreader, and evaporated to leave a thin layer of DNA. Covalent surface modification was then initiated by ultraviolet irradiation with a mercury arc lamp through a mask with an aligner for 1 min, resulting in DNA patterning only in regions corresponding to transparent regions of the mask. The DNA species that did not react with the surface were then removed by rinsing with a 0.4% aqueous solution of sodium docecyl sulfate, followed by deionized water. The DNA-modified surface was then blocked for 30 min with a 1% solution of bovine serum albumin in Dulbecco's phosphate buffered saline to minimize nonspecific interactions. Subsequent introduction of *Saccharomyces cerevisiae* modified to display the complementary DNA strand[23] allowed for efficient and rapid patterning of cells only on the regions of the slide that were exposed to the light source, as shown in Figure 4.

A number of control experiments were performed to confirm the DNA-dependent nature of the patterning (Figure 4c). No DNA was deposited in the nonilluminated areas, and the unreacted aniline groups did not bind the DNA-coated cells (especially after BSA blocking). In addition, no patterning was observed after irradiation in the presence of noncomplementary strand **6e** ("mismatched") or after the irradiation of glass modified with nonreactive benzylalkoxyamine **15** in place of aniline alkoxyamine **13**. Analogous control experiments for glass modified with silane **14** yielded similar results (Figure S14b).

A significant advantage of this approach is the ability to photopattern complex features quickly, as demonstrated by the generatation of several patterns of living yeast cells using masks during the irradiation steps. Figure 4d exemplifies the ability to generate accurate arbitrary patterns with minimal levels of background binding. In addition, Figures 4e–g show the ability of the method to generate feature sizes as small as 20 μm while maintaining a high degree of cell binding. Aniline glass prepared using the oxime formation or through the use of silane **14** and subsequent TCEP reduction functioned equivalently in these experiments.

An additional attractive feature of this new patterning method is its efficient use of the DNA-azidophenol reagent, which becomes increasingly important for the coverage of large slide areas. In our previous work, we have used a reductive amination method to attach amine-labeled DNA to aldehyde-coated glass.[20−26] Those optimized conditions require 1 nmol of DNA reagent to cover 1 cm^2 of slide, and even this amount leads to incomplete coverage when applied to large areas. Due to the higher efficiency of this photochemical method, as little as 128 pmol of DNA can be used to cover the same 1 cm^2 area. Importantly, significantly greater cell pattern density can be achieved despite the lower quantity of reagent that is applied (for example, see Figure 4f).

The described technique allows for the precise patterning of DNA in <1 h using a standard mask aligner or UV hand lamp. The simplicity of the technique and synthetic accessibility of the reagents make it a good candidate for widespread adoption and application. The demonstrated use of this method in combination with DNA-mediated cell patterning allows for significantly faster and more precise immobilization of cells with respect to the current state of the art. This UV-initiated reaction provides a robust solution to patterning that is not easily achieved using current techniques and has great potential for simple and rapid fabrication of spatially resolved, multifunctional surfaces.

■ CONCLUSION

In summary, we have developed a new photoinitiated reaction involving azidophenols and anilines, and we have demonstrated its utility for the photopatterning of DNA strands on surfaces with subsequent cell immobilization. We are currently developing azidophenol analogs with longer wavelength sensitivity to broaden substrate compatibility. The general nature of this method should facilitate its use in a variety of applications, and our lab is currently focusing on extending the scope of this technique to include the rapid generation of multistrand DNA patterns for applications in cell and enzyme patterning. As we have previously demonstrated the use of DNA as a templating architecture for a variety of cell types, including adherent and nonadherent mammalian cells, we envision using this technique to study cellular interactions resulting from the patterning of multiple cell types in close proximity. Finally, the technical simplicity and large number of substrates already demonstrated with this method (i.e., small molecules, polymers, proteins, and nucleic acids) suggest that it could provide a broadly applicable solution to photopatterning.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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