Fast Surface Immobilization of Native Proteins through Metal-Free Amino-yne Click Bioconjugation

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In this work, we use a spontaneous and catalyst-free amino-yne click bioconjugation to generate activated ethynyl group functionalized surfaces for fast immobilizing native proteins and cells. Biomolecules, such as bovine serum albumin (BSA), human IgG and peptide of C(RGDfK), could be covalently immobilized on the surfaces in as short as 30 min. Notably, the bioactivity of the anchored biomolecules remain intact, which is verified by efficiently capturing target antibodies and cells from the bulk solutions. This strategy represents an alternative for highly efficient surface biofunctionalization.

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Fast Surface Immobilization of Native Proteins through Metal-free Amino-yne Click Bioconjugation

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Supporting Information Placeholder

ABSTRACT: Surface immobilization provides a useful platform for biosensing, drug screening, tissue engineering and other chemical and biological applications. However, some of used reactions are inefficient and/or complicated, limiting their applications in immobilization. Herein, we use a spontaneous and catalyst-free amino-yne click bioconjugation to generate activated ethynyl group functionalized surfaces for fast immobilizing native proteins and cells. Biomolecules, such as bovine serum albumin (BSA), human IgG and peptide of C(RGDfK), could be covalently immobilized on the surfaces in as short as 30 min. Notably, the bioactivity of the anchored biomolecules remain intact, which is verified by efficiently capturing target antibodies and cells from the bulk solutions. This strategy represents an alternative for highly efficient surface biofunctionalization.
that enjoys the advantages of 100% atom efficiency, mild reaction and metal-free conditions, and stereo- and regioselectivity, etc.\textsuperscript{[28-32]} More recently, the amino-yne click reaction was proved to hold great potentials for bioconjugation applications.\textsuperscript{[33,34]} Attracted by this highly efficient spontaneous amino-yne click reaction, herein, we applied it in functionalizing the surfaces and sequentially immobilizing bioconjugates for the first time. Excitingly, the amino group coated glass surfaces could be successfully functionalized with activated ethynyl groups through the catalyst-free amino-yne click reaction, which could be further modified with organic dyes, native proteins and cells in one step (Figure 1). Furthermore, we combined the amino-yne click reaction with micro contact printing (µCP) technique to seed HeLa cells and mesenchymal stem cells (MSCs) on the surfaces with well-defined patterns, which is promising for application in tissue engineering.

The glass slides were chosen as the model substrate because of the wide applications in bioassay. Briefly, the

![Diagram](image)

**Figure 1.** The illustration of spontaneous amino-yne click reaction (A) and (B) and its application in surface biofunctionalization (C).

glass surfaces were first treated with aminopropyltriethoxysilane (APTES) to readily generate amino group coated surfaces,\textsuperscript{[34]} followed with the excessive addition of activated diyne 1 (Figure 2A). Then, an activated ethynyl group coated layer on the glass surface was fabricated. To verify the reaction proceeding between activated ethynyl groups of diyne 1 and amino groups on the surface, we studied the reaction of APTES with diyne 1 in tetrahydrofuran (THF) solution by using the Fourier-transform infrared spectroscopy (FT-IR). The results showed that a new peak at 1610 cm\(^{-1}\) corresponding to the newly formed vinyl groups appeared (Figure S1), indicative of the successful reaction of APTES with diyne 1.\textsuperscript{[30]} This result suggests that the ethynyl groups of 1 can react with amino groups of APTES on the surface effectively.

To further confirm the success of the modification, the water contact angles of surfaces with different treatments were measured (Figure 2A). As listed in Table S1, the distinct change in hydrophobicity could be observed after the silylation treatment towards the surface. The subsequent reaction of APTES with diyne 1 further increased the hydrophobicity of the surface (\(\Delta \theta\) from 9.55° to 18.42°) probably due to the introduction of the hydrophobic alkyl chains. Meanwhile, X-ray photoelectron spectroscopy (XPS) measurements of the surfaces before and after the modification of diyne 1 further proved the occurrence of amino-yne click reaction on the surfaces. As shown in Figure S2, new peak assignable to the C-O band appeared after the treatment of diyne 1 with surface 2. Furthermore, the data of element percent ratio of C/N revealed that the values of surface 2 increased compared to that of surface 3 (Table S2). Based on above analysis, we could conclude that the activated ethynyl groups have been covalently introduced on the surfaces of glass slides.

With the activated ethynyl groups modified surfaces in hand, we treated them with functional molecules through the amino-yne click reaction. First, hydroxyl-, ethynyl- and amino-containing luminogens 2, 3 and 4 (Figure 2B) were designed and synthesized (Schemes S1-S3). Notably, these luminogens feature the unique aggregation-induced emission (AIE) characteristics (AIEgens) (Figure S3). According to the AIE mechanism of restriction of intramolecular motion (RIM)\textsuperscript{[39]}, once the AIEgen was immobilized on the surface, it would be emissive upon irradiation. We then treated the surfaces 1-3 with the AIEgens of 2-4, respectively. As shown in Figure 2C and 2D, intense fluorescence can only be detected on the surfaces 2 and 3 upon treating with 3 and 4 under UV lamp irradiation after general washing process with THF. This phenomenon could be well explained: AIEgen 3 could covalently immobilized on the amino-functionalized surface 2, whereas, 4 could be
covalently bound on the ethynyl-containing surface 3 through the spontaneous amino-yne click reaction, and the RIM mechanism enabled the surfaces emissive. However, the treatment of 2-4 with surface 1, 2 and 4 with surface 2 as well as 2 and 3 with surface 3 resulted in no emission upon UV irradiation because the AIEgens could be washed away due to the noncovalent linkage between the molecules and the surfaces. These results further confirmed that ethynyl groups had been well-functionalized on the surface 3, and the amino-yne click reaction could be performed successfully for immobilization applications.

Next, we tried to immobilize native proteins on the ethynyl group functionalized surface via amino-yne click reaction (Figure 3A). Bovine serum albumin (BSA) was firstly employed due to its wide applications in surface biomodification. The ethynyl group coated surface was incubated with FITC-labeled BSA (BSA-FITC) followed with washing to remove the unreacted ones. Then, the immobilization rate of BSA was evaluated by Gel imaging system. As shown in Figure 3A, strong fluorescence signal on the surface was observed after treatment with BSA-FITC. Time-lapse imaging of immobilization process of BSA-FITC on the surface indicated that the reaction could complete within 30 min (Figure 3B). Moreover, by increasing the concentration of BSA-FITC, the fluorescence intensity of the formed spots on the surface was enhanced (Figure 3C) under the same treatment conditions because more BSA-FITC could be immobilized on the surface.

Encouraged by the successful and efficient immobilization of BSA on the ethynyl group coated surface, we further tried to fabricate a functional biochip, and human IgG was selected as a model antibody. We first investigated its immobilization efficiency and bioactivity on the surface. After spotting the human IgG solution on the surface 3 for 30 min at room temperature, the functionalized surface was washed with PBS buffer containing 0.2% sodium dodecyl sulfate to remove unreacted antibody, and then blocked with BSA to inhibit the nonspecific adsorption. Next, we added Cy5-labeled goat anti-human IgG (Goat Anti-human IgG/Cy5), the secondary antibody, on the surface to test the bioactivity of human IgG. As shown in Figure 3D, by increasing the concentration of antibody, fluorescent signal of the spots increased synchronously. As a sharp contract, the sole BSA coated surface showed no observable fluorescent signal after treatment with the antibody under the same experimental conditions (Figure S4). This result well excluded the non-specific physical adsorption of secondary antibody on the surface.

Furthermore, we tried to immobilize cells on the surface because of the important roles of cell adhesion in tissue engineering. Herein, MSCs were chosen for surface adhesion due to its broad applications in bone regeneration. To achieve this goal, C(RGDfK), a short peptide that can enhance cell adhesion through integrin-mediated interaction, was first immobilized on the surface 3 via amino-yne click reaction. Then, MSCs were incubated with C(RGDfK) modified surface for 3 h followed by mild washing process. For comparison, surfaces 1-3 were also used to treat with MSCs under the same conditions. As shown in Figures 3E and S5, only the C(RGDfK) modified surface could greatly promote the adhesion of MSCs. In addition, the cells on both the ethynyl group and C(RGDfK) functionalized surfaces
exhibit normal cell morphology, indicative of the good biocompatibility of these two kinds of surfaces.

It has been demonstrated that certain patterns can promote the differentiation of MSCs.\(^{36}\) To further exploit the versatility of amino-yne click reaction in surface modification, we combined micro contact printing (\(\mu\)CP) technique with amino-yne reaction to form well defined functional patterns on the surface (Figure 4A). The patterned surfaces were prepared using the similar procedures as previous process except that the APTES reagents were transferred to the glass slides via the mediation of PDMS stamps. HeLa cells were incubated with the patterned surface, and the results showed that they could selectively adsorb on the patterned surfaces (Figure 4B, C and Figure S6). Similar results were observed by using MSCs for pattern preparation (Figure 4D, E, and Figure S6), although the adhesion efficiency was not as high as that of HeLa cell because generally HeLa cell has a bigger adhesion force than MSCs.\(^{38}\)

In conclusion, we generate new type of surfaces for biomolecule immobilization by using the efficient and spontaneous amino-yne click reaction. This kind of ligation tool does not require catalyst or harsh reaction conditions, and the reaction between ethynyl group coated surfaces and biomolecules can be finished within 30 min. Through this technique, we successfully immobilize proteins and cells on the surfaces with their bioactivity intact. Moreover, we also use \(\mu\)CP technique and amino-yne click reaction to seed cells on the surfaces with the well-defined patterns. The proposed technique and the generated surfaces in this work exhibit good reactivity, biocompatibility,
simple operation, and hold great potential in surface biofunctionalization.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.

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Notes
The authors declare no competing financial interest.

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Supporting Information

Fast surface immobilization of native proteins through metal-free amino-yne click bioconjugation

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Materials and instruments:

All chemicals and reagents were purchased from commercial sources and used as received without further purification. $p$-Toluene sulphonic acid was purchased from Beijing HWRK Chem Co. Ltd (China). Toluene was purchased from Sigma. (3-Aminopropyl)triethoxysilane, 4-bromobenzophenone, diphenylamine, $N$-(4-bromobutyl)phthalimide and potassium carbonate were purchased from Energy Chemical (China). Sodium tert-butoxide, 1,6-hexanediol and 4-hydroxybenzophenone were purchased from TCI. Propiolic acid was purchased from Acros. Tetrahydrofuran (THF), Titanium tetrachloride and Zinc was purchased from Aldrich. Acetonitrile was purchased from J&K. Water was purified with a Millipore filtration system. C(RGDfK) was purchased from GL Biochem, Shanghai. BSA was purchased from Aladdin. BSA-FITC, Human IgG and Goat Anti-human IgG/Cy5 were purchased from Ruixi, Xi’an. Contact angle were measured on POWEREACH, X-ray photoelectron spectroscopy (XPS) were measured on a Kratos Axis Ultra DLD, Fourier transform infrared (FT-IR) spectra were measured on a Bruker Vector 33 FT-IR spectrometer (KBr disk). NMR spectra were measured on a Bruker AV 500 spectrometer. Photoluminescence spectra were recorded on a Horiba Fluoromax-4 spectrofluorometer. Absorption spectra were recorded on SHIMADZU. Fluorescent images were taken by Carl Zeiss Axio Vert.A1.

The preparation of ethynyl group functionalized surface

Glass substrates were firstly cleaned by sonication in ethanol and water, sequentially, followed with the drying under a stream of $N_2$. The substrates were then treated with freshly prepared piranha solution (3:1 $H_2SO_4/H_2O_2$ ; Caution!) for 1 h at 100 °C. Then, the substrates were washed with water to remove the sulfuric acid and dried under a stream of $N_2$. The cleaned substrates were immersed in a solution of 3-aminopropyltriethoxysilane (APTES, 5 vol %) in ethanol for 24 h and then washed with ethanol, followed with the drying under a stream of $N_2$. The dry substrates were then annealed at 120 °C for 1 h to produce covalently grafted amine-coated substrates.
After heating, the substrates were washed by methylbenzene for three times with the assistance of ultrasonic and dried under N\textsubscript{2}. Amine-functionalized substrates were converted to ethynyl group-functionalized substrates by immersing the former into a solution of diyne 1 (50 mg/mL) in THF for 5 h at room temperature. Finally, the substrates were washed with THF, ethanol and water subsequently with the assistance of ultrasonic, and were dried with a stream of N\textsubscript{2}.

**Immobilization of BSA, Human IgG on the ethynyl group functionalized surfaces**

For BSA: The ethynyl group functionalized substrate was incubated with FITC-labeled bovine serum albumin in phosphate buffer (pH 7.4, 0.01 M) for 30 min at room temperature. The substrate was then washed with 0.2% SDS solution and water to remove the unreacted BSA.

For Human IgG: The ethynyl group functionalized substrate was treated with the Human IgG in phosphate buffer (pH 7.4, 0.01 M) for 30 min at room temperature in a wet box. After washing with 0.2 % SDS and water, the surface immobilized with the Human IgG was further blocked by BSA (1 mg/mL), and was incubated with Cy5-labeled goat-anti-human IgG for 30 min at room temperature in a wet box. Finally the slide was washed by 0.2 % SDS and water, sequentially.

**The adhesion of cells**

The ethynyl group functionalized surface was incubated with a solution of C(RGDfK) in DMSO (0.5 mg/mL) for 5 h at room temperature. The unreacted C(RGDfK) was removed by washing with DMSO, ethanol and Milli-Q water subsequently. Then, cells (1.2 × 10\textsuperscript{5} cells/mL) were seeded respectively on the C(RGDfK) modified surface and cultured for 3 h, then the surfaces were rinsed with PBS buffer for 3 times to remove the unattached cells. The adhesion of cells for the surface functionalized with hydroxyl-, amino- or ethynyl group were operated with the same process as that of the ethynyl group functionalized surface.

**Preparation of cell patterns**

The PDMS stamp was inked with a solution of APTES (1 vol%, 10 μL) in ethanol
for 1 min under a cleaned coverslip. The stamp was rinsed with ethanol for 10 s and then dried with a strong stream of N\textsubscript{2} gas. PDMS stamp was then brought into contact with piranha activated glass slides for 15 s. The slides were then heated at 120 °C for 1 h on a hot plate to enable the formation of covalent siloxane bond between the silanols and the hydroxyl groups on the glass surface. Then the substrate was immersed in the THF solution of 1 (50 mg/mL) for 5 h at room temperature. After subsequently washed by THF, ethanol, water and dried in a stream of N\textsubscript{2}, the slides were incubated with a solution of C(RGDfK) in DMSO (0.5 mg/mL) for 5 h at room temperature to form the C(RGDfK) patterned surfaces. Then, they were put into Petri dish and exposed under UV for 30 min. The surface was incubated with the solution of HeLa cells (1.2 \times 10^5 cells/mL) or MSCs for 24 h at 37 °C, respectively. Finally, the surface was vigorously washed with PBS buffer to obtain the patterns.

**The preparation of compound 2.**

Compound 5 (1.7 g, 5 mmol), 6 (4 g, 20 mM), and zinc powder (4 g, 60 mmol) were added into a 250 mL two-necked round bottom flask equipped a condenser. The flask was evacuated under vacuum and flushed with dry nitrogen three times, followed with the addition of 100 mL of THF. After cooling to -78 °C, TiCl\textsubscript{4} (4 mL, 30 mmol) was injected dropwise into the mixture. The mixture was slowly warmed to room temperature and stirred overnight. The mixture was quenched with 10% aqueous Na\textsubscript{2}CO\textsubscript{3} solution and filtered. The filtrate was extracted with ethylacetate three times. The organic layer was washed with water and dried over anhydrous magnesium sulfate. After filtration and solvent evaporation, the crude product was purified by silica gel column chromatography to afford a yellow solid in 35% yield. (FT-IR (v, cm\textsuperscript{-1}): 3666, 3305, 1693, 1588, 1283. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 7.21 (m, 4H), 7.13-6.97 (m, 16H), 6.89-6.76 (m, 6H), 6.61 (d, 1H), 6.54 (d, 1H), 4.53 (s, 1H). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \(\delta\) 153.86, 147.74, 147.57, 145.78, 144.16, 143.81, 139.88, 138.11, 136.41, 132.74, 132.13, 131.38, 131.36, 129.13, 129.08, 128.42, 127.68, 127.56, 126.26, 124.21, 124.14, 123.70, 122.72, 122.60, 114.52, 114.47.)

**The preparation of Compound 3.**

To a solution of compound 2 (210 mg, 0.41 mmol) and propiolic acid (25mg, 0.37
mmol) in DCM (10 mL) at 0 °C, a combined solution of DCC (76 mg, 0.37 mmol) and DMAP (4.5 mg, 0.037 mmol) in DCM (10 mL) was added slowly dropwise and stirred for 5 h. The reaction mixture was filtered and washed with diethyl ether. The reaction mixture was washed with 1 M NaOH, water, brine, and dried over Na₂SO₄.

After filtration the solvent was evaporated and the crude residue was purified by column chromatography (yield: 18%). (FT-IR (v, cm⁻¹): 2127, 1735, 1591, 1484, 1179. ¹H NMR (500 MHz, CDCl₃) δ 7.23-7.22 (m, 4H), 7.19-6.95 (m, 20H), 6.89-6.77 (m, 6H), 3.02 (d, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 150.72, 148.23, 148.14, 147.50, 146.22, 146.12, 143.64, 143.34, 143.25, 142.49, 142.19, 141.48, 139.20, 137.52, 137.29, 132.47, 132.10, 131.36, 129.14, 127.81, 127.71, 126.67, 126.55, 124.43, 122.76, 122.46, 120.32, 29.57.)

**The preparation of Compound 4.**
Compound 2 (2.06 g, 4 mmol), 2-(4-bromobutyl)isoindoline-1,3-dione (1.13 g, 4 mmol) and anhydrous potassium carbonate (1.66 g, 12 mmol) were added into 60 mL acetonitrile in a 250 mL three-necked flask and were heated to reflux for 20 h under a nitrogen atmosphere. After the reaction was cooled to room temperature, the solvent was evaporated and the crude product was purified by column chromatography using ethyl acetate/petroleum ether (1:9, v/v) to obtain compound 7 as a yellow solid (yield: 87%). To a solution containing compound 7 (2.5 g, 4.5 mmol) in anhydrous ethanol (100 mL), excess hydrazine hydrate (4.5 g, 90 mmol, 20 eq.) was added dropwise. The resulting mixture was stirred at room temperature for 12 h. A lot of white solid was precipitated and filtered. Then the filtrate was concentrated under vacuum and obtained compound 4 as yellow solid (yield: 65%). (FT-IR (v, cm⁻¹): 3057, 1596, 1506, 1281, 1176.) ¹H NMR (400 MHz, d-DMSO) δ,7.28-7.24 (m, 4H), 7.20-7.08 (m, 6H), 7.04-6.91 (m, 10H), 6.87-6.80 (m, 4H), 6.76-6.66 (m, 4H) 3.91-3.85 (m, 2H), 2.57-2.50 (m, 2H), 1.73-1.60 (m, 2H), 1.48-1.38 (m, 2H). ¹³C NMR (125 MHz, CDCl₃) δ 157.49, 157.39, 147.76, 147.58, 145.74, 144.29, 144.03, 143.87, 140.05, 139.71, 138.25, 136.30, 136.05, 132.53, 132.14, 131.40, 129.10, 127.68, 127.49, 126.21, 124.12, 123.61, 122.84, 122.60, 122.23, 114.39, 113.93, 113.40, 67.42, 41.87, 30.26, 26.70.)
Scheme S1. Synthetic route to AIEgen 2.

Scheme S2. Synthetic route to AIEgen 3.

Scheme S3. Synthetic route to AIEgen 4

Table S1. The characterization of static ($\theta_s$), advancing ($\theta_{adv}$), receding ($\theta_{rec}$) water contact angles and $\Delta\theta$ ($\theta_{adv}-\theta_{rec}$).

| surface          | $\theta_s$ (°) | $\theta_{adv}$ (°) | $\theta_{rec}$ (°) | $\Delta\theta$ (°) |
|------------------|----------------|---------------------|--------------------|-------------------|
| Uncleared slide  | 41.50 ± 2.21   | 41.35 ± 3.02        | 29.98 ± 2.24       | 11.37 ± 2.64      |
| Surface 1        | 8.90 ± 0.21    | 10.54 ± 1.05        | 8.08 ± 0.79        | 2.46 ± 0.92       |
| Surface 2        | 73.32 ± 0.41   | 73.63 ± 1.36        | 64.08 ± 1.03       | 9.55 ± 1.20       |
| Surface 3        | 66.92 ± 1.16   | 72.26 ± 1.19        | 53.84 ± 0.72       | 18.42 ± 0.47      |
Table S2. The atomic percentage of elements on the Surfaces 2 and 3 based on XPS.

| Surface   | Element mole percent (atom%) | C  | N  | O  | Si | C/N |
|-----------|------------------------------|----|----|----|----|-----|
| Surface 2 |                              | 24.75 | 3.47 | 47.50 | 24.28 | 7.133 |
| Surface 3 |                              | 64.21 | 5.96 | 20.78 | 9.05 | 10.773 |

Figure S2. FT-IR spectra of (A) APTES, (B) diyne 1 and (C) compound 9.

Figure S3. XPS characterization of Surface 2 and Surface 3.
**Figure S1.** Absorption spectra of 2 (A), 3 (B) and 4 (C). Emission spectra of 2 (D), 3 (E) and 4 (F) in THF and THF/water mixtures. Concentration: 1 μM. λ<sub>ex</sub>: 344 nm.
Figure S4. The comparison of antibody capture efficiency between human IgG and BSA modified surfaces. $\lambda_{ex} = 649$ nm.

Figure S5. MSCs adhesion behaviors on surface 1 (A), surface 2 (B), and surface 3 (C). For all of the surfaces, the adhesion time is 3 h. Scale bar is 100 μm.
Figure S6. Region-selective adhesion behavior of two cells on the substrate. (A) and (B) are HeLa cells. (C) and (D) are MSCs. $\lambda_{ex} = 490$ nm.

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