Electronic Supplementary Information (ESI)

Fast Surface Immobilization of Native Proteins through Catalyst-Free Amino-Yne Click Bioconjugation

Yiru Zhang,[‡a] Jianlei Shen,[‡a] Rong Hu,[a] Xiujuan Shi,[b] Xianglong Hu,[b] Benzhao He,[b] Anjun Qin,*[a] and Ben Zhong Tang*[ab]

[a] State Key Laboratory of Luminescent Materials and Devices, Guangdong Provincial Key Laboratory of Luminescence from Molecular Aggregates, Center for Aggregation-Induced Emission, South China University of Technology, Guangzhou 510640, China.

[b] Department of Chemistry, Hong Kong Branch of Chinese National Engineering Research Centre for Tissue Restoration and Reconstruction, Institute for Advanced Study, and Department of Chemical and Biological Engineering, The Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong, China

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-bromo-butyl)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 Ulra 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.

Preparation of ethynyl group functionalized surface

Glass substrates were first 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 than annealed at 120 °C for 1 h to produce covalently grafted amine-coated ones. After heating, the substrates were washed by methylbenzene for three times with the assistance of ultrasonic and dried under N_2. Amine-functionalized substrates were

converted to ethynyl group-functionalized ones 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_2.

Immobilization of BSA, Human IgG on ethynyl group functionalized surfaces

Immobilization of BSA: The ethynyl group functionalized substrate was incubated with FITC-labeled bovine serum albumin (BSA-FITC) 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.

Immobilization of 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.

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 (1.2×10^5 cells/mL) were seeded respectively on the C(RGDfK) modified surface and cultured for 3 h. Afterward, the surfaces were rinsed with PBS buffer for 3 times to remove the unattached cells. The adhesion of cells for the surfaces functionalized with hydroxyl-, amino- or ethynyl group were operated with the same processes 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_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_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 (1.2×10^5 cells/mL) or MSCs for 24 h at 37 ℃, respectively. Finally, the surface was vigorously washed with PBS buffer to obtain the patterns.

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 cooled to -78 °C, $TiCl_4$ (4 mL, 30 mmol) was injected dropwise into the mixture. After finishing the addition, the mixture was slowly warmed to room temperature and stirred overnight, and then quenched with 10% aqueous Na_2CO_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 using petroleum ether (PE)/DCM as eluent with gradually increasing the ratio of DCM. A yellow solid was obtained in 35% yield. FT-IR (v, cm^{-1}): 3666, 3305, 1693, 1588, 1283. ^1H NMR (500 MHz, $CDCl_3$) δ 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). ^{13}C NMR (125 MHz, $CDCl_3$) δ 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.

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)

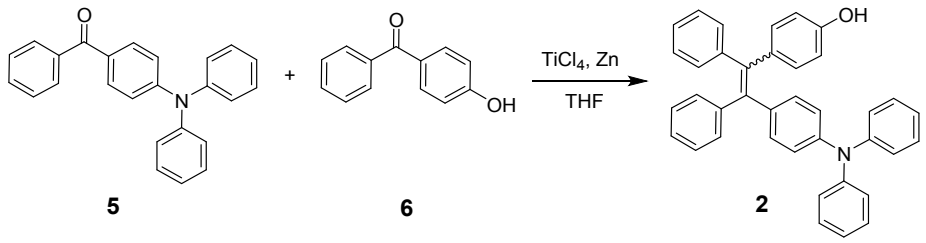
and DMAP (4.5 mg, 0.037 mmol) in DCM (10 mL) was added dropwise and stirred for 5 h. The reaction mixture was filtered and washed with diethyl ether. The reaction mixture was washed with 1 M NaOH, water, brine, and dried over Na_2SO_4. After filtration and solvent evaporation, the crude product was purified by silica gel column chromatography using PE/DCM (5:1, v/v) as eluent. A yellow solid of 3 was obtained in 18% yield. FT-IR (ν, cm^{-1}): 2127, 1735, 1591, 1484, 1179. ^1H NMR (500 MHz, $CDCl_3$) δ 7.25-7.20 (m, 4H), 7.16-6.94 (m, 18H), 6.89-6.77 (m, 6H), 3.02 (d, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) 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.

Preparation of compound 4.

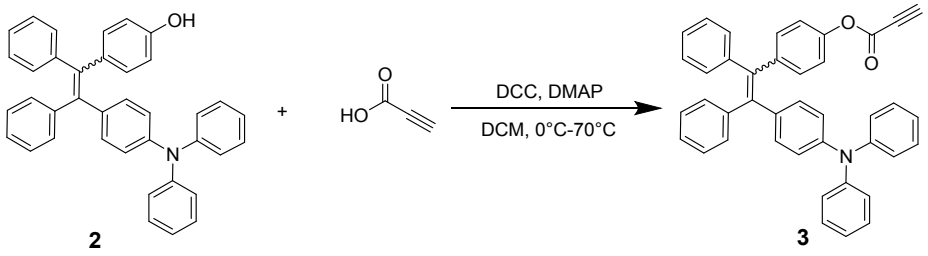
Compound **2** (2.06 g, 4 mmol), 2-(4-bromobutyl)isoindoline-1,3-dione (1.13 g, 4 mmol) and anhydrous potassium carbonate (1.66 g, 12 mmol) were added into 60 mL acetonitrile in a 250 mL three-necked flask and were heated to reflux for 20 h under an nitrogen atmosphere. After the reaction mixture was 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), and compound **7** was obtained as a yellow solid in 87% yield.

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 resultant 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 compound **4** was obtained as yellow solid in 65% yield. FT-IR (ν, cm^{-1}): 3057, 1596, 1506, 1281, 1176.) ^1H NMR (400 MHz, DMSO-d_6) 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). ^{13}C NMR (125 MHz, $CDCl_3$) 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,

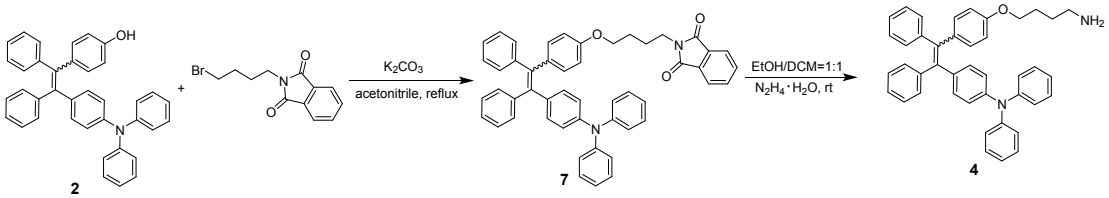
30.26, 26.70.



Scheme S1. Synthetic route to AIEgen **2**.



Scheme S2. Synthetic route to AIEgen **3**.



Scheme S3. Synthetic routes to AIEgen **4**.

Table S1. Comparison of different methods for surface immobilization application.

Functional group	Interaction	Advantage	Disadvantage	Ref
Activated ethynyl	Covalent ligation	Easy fabrication Rapid and stable linkage Short reaction time (30min) Catalyst-free feature and room temperature No protein modification requirement. No background fluorescence	Non-specific protein immobilization	This work
Aldehyde	Covalent ligation	Easy fabrication No protein modification requirement Catalyst-free feature and room temperature	Non-specific protein attachment The linkage is unstable and always needs to be firmed by reducing agent Background fluorescence at 488 nm Long reaction time (>3 h)	1, 2
Nitrilotriacetic acid (NTA)	Affinity binding	Specific protein attachment Low background	Hard fabrication Proteins need to be Hisx6-tagged. Linkage is relatively weak	3
Epoxy	Covalent ligation	Rapid and stable linkage Reaction time (1 h) No protein modification requirement	Non-specific protein attachment High ionic reaction medium is required which may cause denaturation of proteins	4
Ethynyl	Covalent ligation	Specific protein attachment Low background	Hard fabrication Proteins need to be modified by azido groups Cu-catalysts are necessary Long reaction time (12 h)	5
Azide	Covalent ligation	Specific protein attachment. Low background.	Hard fabrication Proteins need to be modified by ethynyl groups Cu-catalysts are necessary Long reaction time (> 2 h)	6, 7
Thiol	Covalent ligation	Specific protein attachment Rapid and stable linkage Reaction time (10 min)	Proteins need to be modified by alkenyl groups UV treatment is required	8
Phosphinothioester	Covalent ligation	Specific protein attachment Rapid reaction and high yield Reaction time (<1min)	Proteins need to be modified by azido groups	9
Maleimide	Covalent ligation	Specific protein attachment Catalyst-free feature and room temperature	Protein needs to be modified by diene. Long reaction time: 8h	10
Fischer carbene	Covalent ligation	Rapid reaction and high yield Reaction time (10min) No protein modification required Catalyst-free feature and room temperature	Non-specific protein attachment. Hard fabrication	11
Carboxy	Covalent ligation	No protein modification required. Catalyst-free feature and room temperature	Non-specific protein attachment Pre-activation by NHS before the immobilization is necessary Long reaction time (2 h)	12
Aniline	Covalent ligation	Specific biomolecules attachment Rapid and stable linkage Short reaction time (1 min) Biomolecular photopattern could be realized	Biomolecules need to be modified by ortho-Azidophenols	13

Table S2. The characterization of static (θ_s), advancing (θ_{adv}), receding (θ_{rec}) water contact angles and $\Delta\theta$ (θ_{adv}-θ_{rec}).

surface	θ_s (°)	θ_{adv} (°)	θ_{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 S3. 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

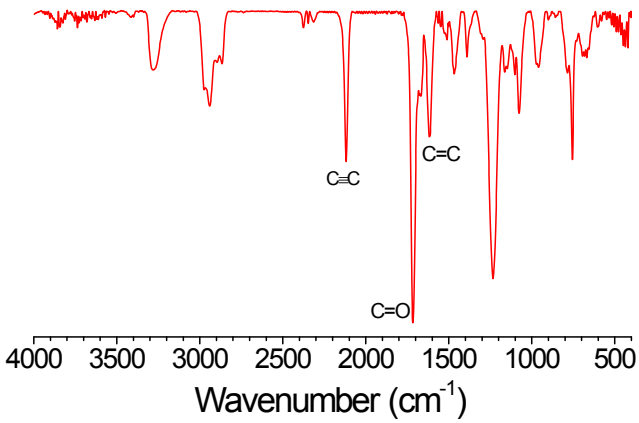


Figure S1. FT-IR spectrum of the product generated from the reaction of APTES with diyne **1** in THF.

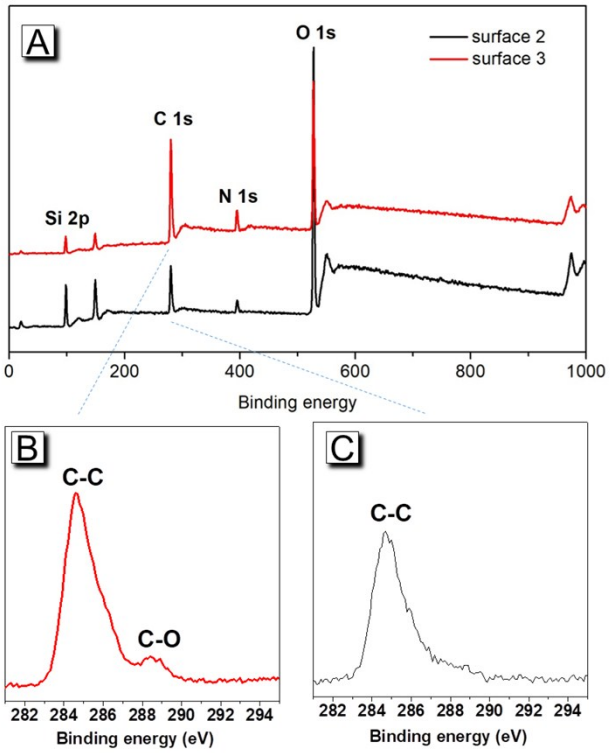


Figure S2. XPS characterization of Surfaces 2 and 3.

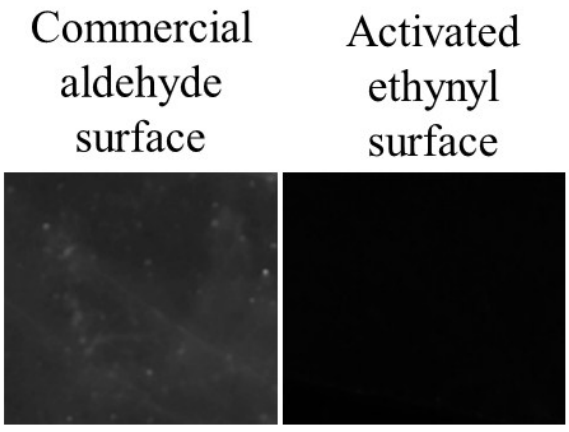


Figure S3. Fluorescent pictures of commercial aldehyde modified surface and activated ethynyl group functionalized surface. λ_{ex} = 488 nm.

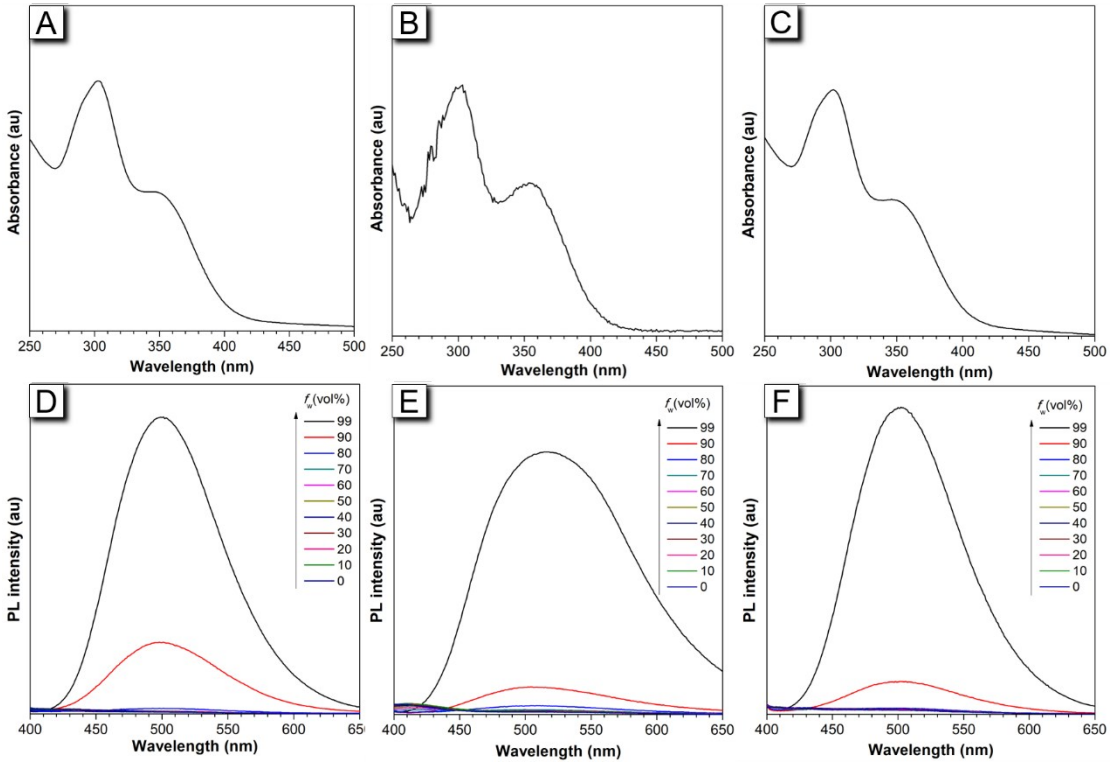


Figure S4. Absorption spectra of **2** (A), **3** (B) and **4** (C) in THF and photoluminescence spectra of **2** (D), **3** (E) and **4** (F) in THF/water mixtures with different water fractions. Concentration: 1 μM. λ_{ex}: 344 nm.

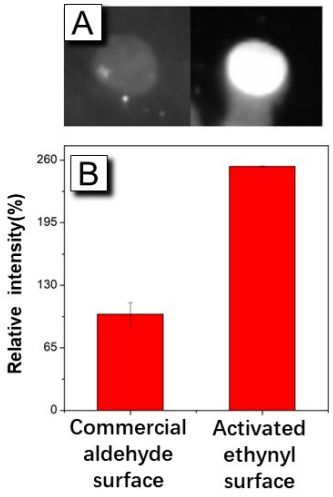


Figure S5. (A) Fluorescent pictures of immobilized BSA-FITC on commercial aldehyde modified surface (left) and activated ethynyl group functionalized surface (right), λ_{ex} = 488 nm. (B) The relative fluorescent intensity of proteins on two kinds of surfaces analyzed by Image J software.

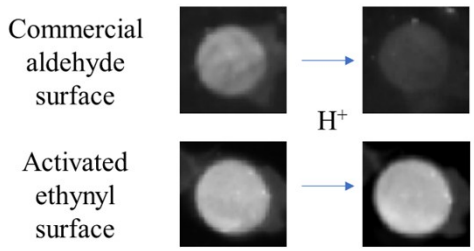


Figure S6. The fluorescent images of commercial aldehyde modified surface and activated ethynyl group functionalized surface immobilized with BSA-FITC before and after treated with buffer solutions containing acetic acid (pH=3) for 1 h. λ_{ex} = 488 nm

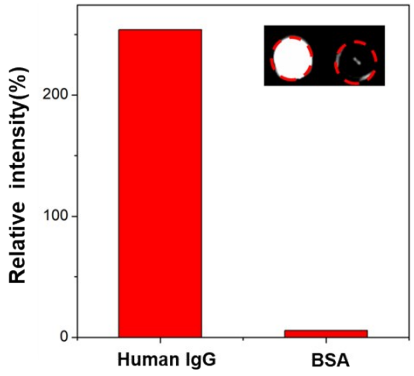


Figure S7. The comparison of antibody capture efficiency between human IgG and BSA modified surfaces. λ_{ex} = 649 nm.

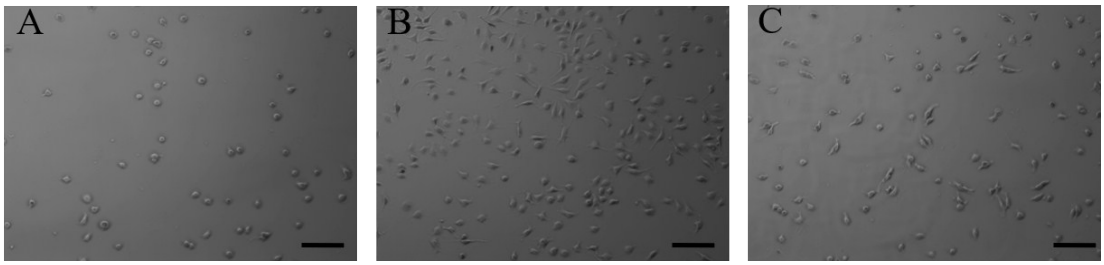


Figure S8. MSCs adhesion behaviors on surface 1 (A), surface 2 (B), and surface 3 (C). For all of the surfaces, the adhesion time is 3 h. Scale bar is 100 μm.

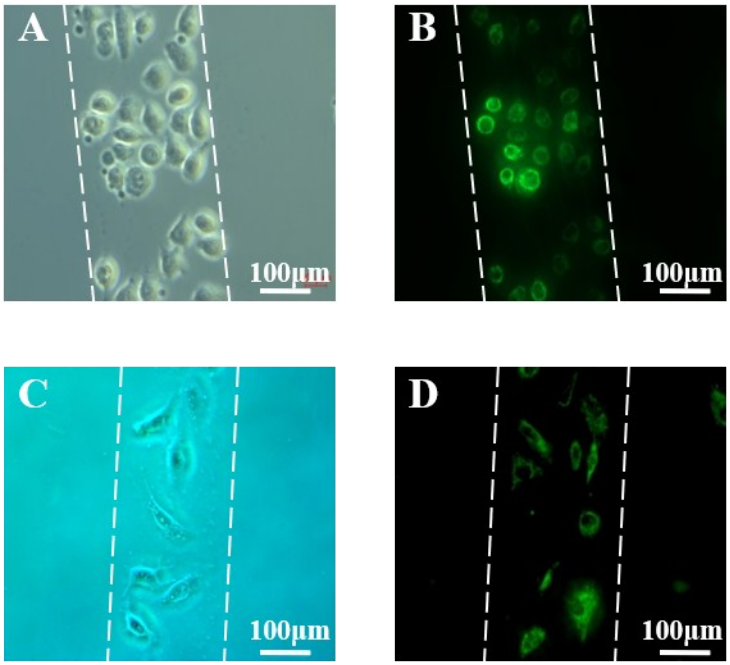


Figure S9. Region-selective adhesion behavior of two cells on the substrate. (A) and (B) are HeLa cells. (C) and (D) are MSCs. λ_{ex} = 490 nm.

References

1 D. Peelen, L. M. Smith, *Langmuir*, 2005, **21**, 266-271.
2 G. MacBeath, S. L. Schreiber, *Science*, 2000, **289**, 1760-1763.
3 N. Haddour, S. Cosnier, C. Gondran, *J. Am. Chem. Soc.,* 2005, **127**, 5752-5753.
4 F. Li, W. Chen, S. Zhang, *Biosens. Bioelectron.* 2008, **24**, 781-786.
5 R. D. Rohde, H. D. Agnew, W. S. Yeo, R. C. Bailey, J. R. Heath, *J. Am. Chem. Soc.,* 2006, **128**, 9518-9525.
6 S. Prakash, T. M. Long, J. C. Selby, J. S. Moore, M. A. Shannon, *Anal. Chem.*, 2007, **79**, 1661-1667.
7 J. S. Seo, S. Lee, D. C. Poulter, *J. Am. Chem. Soc.*, 2013, **135**, 8973-8980.
8 D. Weinrich, P. C. Lin, P. Jonkheijm, U. T. T. Nguyen, H. Schrder, C. M. Niemeyer, K. Alexandrov, R. Goody, H. Waldmann, *Angew. Chem. Int. Ed.,* 2010, **49**, 1252-1257.
9 M. B. Soellner, K. A. Dickson, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.,* 2003, **125**, 11790-11791.
10 A. D. de Araújo, J. M. Palomo, J. Cramer, M. Köhn, H. Schröder, R. Wacker, C. Niemeyer, K. Alexandrov, H. Waldmann, *Angew. Chem. Int. Ed.,* 2006, **45**, 296-301.
11 S. Sawoo, P. Dutta, A. Chakraborty, R. Mukhopadhyay, O. Bouloussac, A. Sarkar, *Chem. Commun.,* 2008, **45**, 5957-5959.
12 S. Herrwerth, T. Rosendahl, C. Feng, J. Fick, W. Eck, M. Himmelhaus, R. Dahint, M. Grunze, *Langmuir*, 2003, **19**, 1880-1887.
13 K. M. El Muslemany, A. A. Twite, A. M. ElSohly, A. C. Obermeyer, R. A. Mathies, M. B. Francis, *J. Am. Chem. Soc.*, 2014, **136**, 12600-12606.