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Supporting Information

Enzymatic Transglycosylation of PEG Brushes by β-Galactosidase

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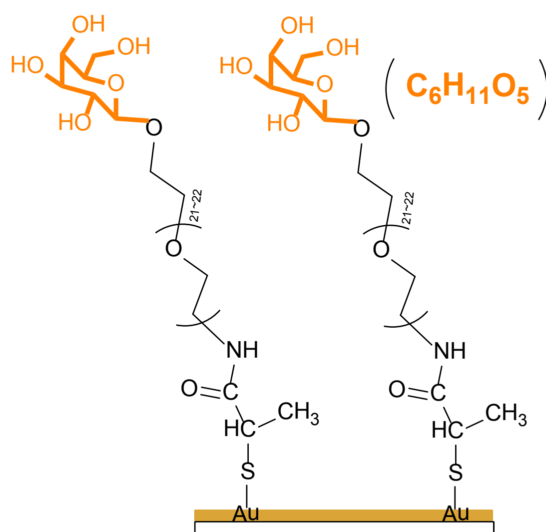


Scheme S1. Idealized construction of the galactose modified PEG brushes on gold.

Materials

3-Mercaptopropionic acid (99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Monoamino-terminated PEG 1000 (NH_2-PEG, 98%) was a commercial product of Shanghai Yare Biotech. Inc. (China). 1-Ethyl-3-(3-di-methylamino-propyl)carbodimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS), β-Galactosidase (β-Gal) from *Aspergillus oryzae* (EC 3.2.1.23), *Ricinus communis agglutinin* (RCA_{120}), fluorescent isothiocyanate-labeled *Ricinus communis agglutinin* (FITC-RCA_{120}) and bovine serum albumin (BSA) were purchased from Sigma Chemical

Co. (St Louis, LA). A 50.0 mM phosphate-buffered saline (PBS, pH 5.5) solution was used as the buffer solution for coupling NH_2-PEG. A 0.1 M citric acid/ sodium citrate buffer (pH 5.0) solution was used as the buffer solution for enzymatic transglycosylation. Water used in all experiments was deionized and ultrafiltrated to 18 MΩ·cm using an ELGA Lab Water system (France).

The commercially available QCM gold sensor chip was comprised of a bare gold surface (Q-sense SX-301, Q-SENSE, Sweden). The chip was rinsed with ethanol and dried under a gentle stream of ultra-pure N_2 gas, after which it was placed in a 1:1:5 mixture of ammonia (28%), hydrogen peroxide (30%), and ultra-pure water, at ~75 ^oC for 10 min. Subsequently, the chip was thoroughly rinsed with ultra-pure water and ethanol, then dried under a steady stream of ultra-pure N_2 gas for further use.

Characterization

Ellipsometry. The variable-angle spectroscopic ellipsometry (VASE) spectra were collected on an MD-2000I spectroscopic ellipsometer (J.A. Woollam, USA) at an incident angle of 60°, 65°, 70° in a wavelength range of 800-1000 nm. A refractive index of 1.45 was assigned to the studied PEG brushes. For data analysis, a two-layer model (Au and Cauchy) was used to calculate the thickness of the brushes. All measurements were conducted in dry air at room temperature. Three separate spots of each sample were measured to obtain a mean brush thickness and associated standard deviation.

Water contact angle (WCA). WCA was determined using a CTS-200 system (Mighty Technology Pvt. Ltd., China) fitted with a drop shape analyzer. Typical experiment was carried out at room temperature by sessile drop method as follows. Briefly, a water drop (2.0 μL) was lowered onto the chip surface from a needle tip. Then, the images of the droplet were recorded. WCAs were calculated from these images with software. At least five different surface locations of each sample were measured and the averaged value was presented.

QCM Monitoring. QCM analysis was carried out using a Q-SENSE E1 system (Q-SENSE, Sweden). The sensor crystals used were 5 MHz, AT-cut, polished quartz discs (chips) with electrodes deposited on both sides (Q-SENSE). The resonance frequency (f) was measured simultaneously at four odd harmonics (5, 15, 25, 35 MHz). In the results, the values reported throughout for Δf was measured at the chosen fifth harmonics. The

working temperature was 25 ^{o}C. Raw data were analyzed with Origin Pro 8.0 (Origin-Lab, USA) and Q-Tools software (Q-SENSE).

Construction of PEG brushes and Characterization. PEG brushes were constructed on QCM chip by a typical procedure. Firstly, 5.0 mM 3-mercaptopropionic acid was dissolved in ethanol and oxygen was removed from the solution by nitrogen bubbling for 5 min. Subsequently, the chip was immersed in the 3-mercaptopropionic acid solution for 12 h to form a self-assembly monolayer (SAM) on the chip surface. The surface was then rinsed in ethanol, dried under a gentle stream of ultra-pure N_2 gas, and immediately assembled into the QCM chamber.

NH_2-PEG was tethered to the chip surface via covalent attachment to the carboxylic acid groups of 3-mercaptopropionic acid SAM. Briefly, the surface carboxylic acid groups were activated as an *N*-hydroxysuccinimidyl ester by EDC (50 mM) and NHS (30 mM) in PBS (pH = 5.5). In the meantime, NH_2-PEG was tethered on the QCM chips through amido linkage formation between the amino end group of NH_2-PEG and the formerly formed activated ester group. Finally, the unreacted activated esters were endcapped with ethanolamine.

As can be seen from Fig. S1, there is about 150 Hz decrease for the QCM frequency during the construction of PEG brushes. In this case, the amounts of the tethered PEG are about 0.2065 ng/cm^2 calculated from QCM data. Thickness of the PEG brushes is 2.12 ± 0.2 nm (determined by ellipsometry) and 6.2 ± 0.8 nm (measured through QCM), respectively. Furthermore, compared to the bare gold surface (WCA is about 53° ± 2°), WCA decreases to 45° ± 2° for the SAM due to the carboxylic acid group of 3-mercaptopropionic acid. After the PEG chains were tethered on the chip surface, the WCA continuously decrease to 40° ± 2°. The result is consistent with the chemical structures of the brushes. Each PEG chain has one hydroxyl end group and about 22~23 ethylene glycol segments (-CH_2CH_2O-). Both of them are contributed to the hydrophilicity of PEG brushes. In addition, a WCA of 36 ± 2° was measured for the galactose-modified PEG brush surface. This character is to be expected from the numerous hydroxyl groups of the galactose unit and the hydrophilicity of PEG chains.

Monitoring of the enzymatic transglycosylation on PEG brushes by QCM. Enzymatic transglycosylation experiments were conducted in real time using a Q-SENSE E1 system (Q-SENSE, Sweden). A peristaltic pump was used to deliver liquids to the channel of the flow cell. A stable baseline signal was established by flowing a 0.1 M citric acid/sodium

citrate buffer (pH 5.0) at a rate of 50 μL/min through the sensor. Then, β-Gal and lactose solutions were injected into the channel, respectively. After enzymatic transglycosylation for a fixed time at 25 °C, PBS buffer was used to remove loosely bound enzymes from the chip surface.

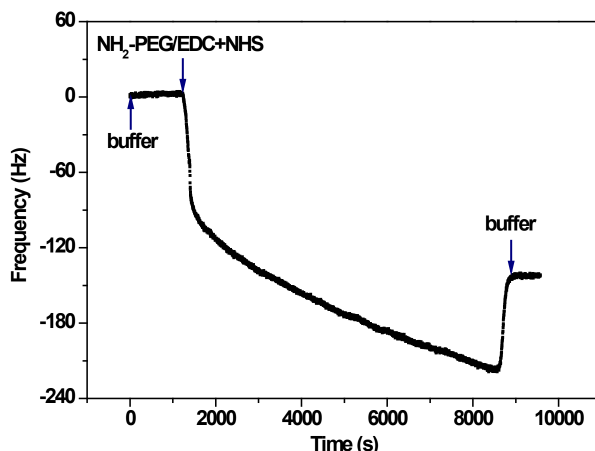
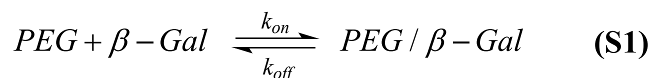


Fig. S1 Typical QCM curve for the construction of PEG brushes on QCM chip.

Binding of β-Gal to the PEG brushes. Previous literature has reviewed that QCM is a useful tool in the detection and quantitative analysis of each step in enzymatic reactions on the surface.[1] In our cases, we can observe three steps continuously from time dependent frequency changes, which include 1) β-Gal binding to PEG brushes; 2) enzymatic transglycosylation on the brushes, and 3) release of the enzyme from the newly constructed galactose-modified brushes. In the first step, β-Gal binding to the hydroxyl acceptor of PEG brushes is described by equation (S1). The amount of PEG/β-Gal complex formed at time *t* after enzyme injection is given by equations (S2) ~ (S4).



$$PEG + \beta - Gal \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PEG / \beta - Gal \qquad \textbf{(S1)}$$

$$[PEG / \beta - Gal]_t = [PEG / \beta - Gal]_\infty \{1 - \exp(-t / \tau)\} \qquad \textbf{(S2)}$$

$$\Delta m_t = \Delta m_\infty \{1 - \exp(-t / \tau)\} \qquad \textbf{(S3)}$$

$$\tau^{-1} = k_{on}[\beta - Gal] + K_{off} \qquad \textbf{(S4)}$$

The relaxation time (τ) of β-Gal binding was calculated from curve fitting the QCM frequency changes at various β-Gal concentrations. β-Gal binding and dissociation rate constants (k_{on} and k_{off}) could be obtained from the slop and intercept of the plot of τ^{-1}

against β-Gal concentration. The binding constant (K_d) could also be obtained from the ratio of k_{on} to k_{off}. Fig. S2 shows typical time courses for β-Gal binding to PEG brushes with changes in the β-Gal concentration. It can be seen that β-Gal-binding amount increase with the increase in β-Gal concentration. According to equation (S4), the obtained k_{on} is 7.41 × 10^{-4} $M^{-1}s^{-1}$, k_{off} is 1.22 × 10^{-3} s^{-1} and K_d is 1.728 M, respectively (Fig. S3).

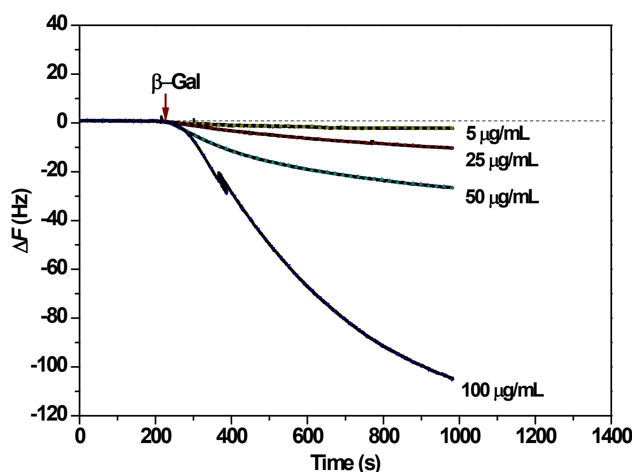


Fig. S2 Binding behavior of β-Gal to PEG brushes dependent on the enzyme concentration.

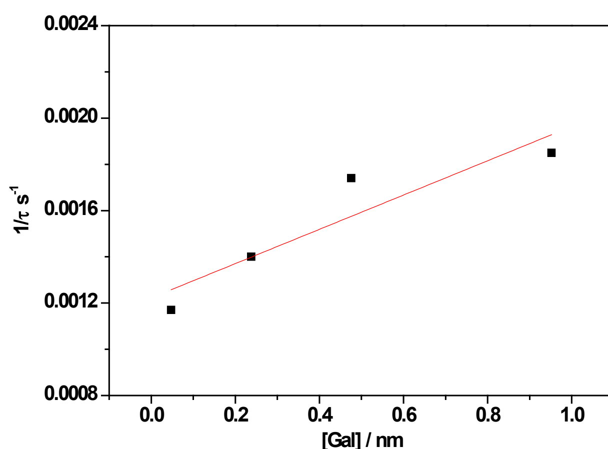


Fig. S3 Linear reciprocal plot of relaxation time τ against β-Gal concentration according to equation S4.

Enzymatic transglycosylation on PEG brushes. Enzymatic transglycosylation occurred by adding lactose after formation of PEG/β-Gal complex on the QCM chip surface. The transglycosylation process is simply expressed by the *Michealis-Menten* model between the PEG/β-Gal complex and the added lactose as shown in equations (S5) ~ (S7), where PEG-galactose indicates the newly prepared galactose-modified PEG brushes. The initial

transglycosylation rate (v_0) increases with the addition of lactose. From the reciprocal plot of v_0 against lactose concentration, the dissociation constant of lactose (k_m) and the catalytic transglycosylation constant (k_{cat}) were obtained from the slope and intercept of the plot.

(S5)

$$v_0 = \frac{k_{cat}[PEG/\beta - Gal]_0[lactose]}{k_m + [lactose]_0} \qquad \textbf{(S6)}$$

$$\frac{1}{v_0} = \frac{k_m}{k_{cat}[PEG/\beta - Gal]_0}\frac{1}{[lactose]} + \frac{1}{k_{cat}[PEG/\beta - Gal]_0} \qquad \textbf{(S7)}$$

XPS analysis. XPS was used to do quantitative element analysis for the modified gold surfaces. XPS spectra were recorded on a PHI-5000C ESCA system (Perkin-Elmer, USA) with Al Kα excitation radiation (1486.6 eV). The pressure in the analysis chamber was maintained at 10^{-6} Pa during measurement. All spectra were referenced to the C_{1s} hydrocarbon peak at 284.6 eV to compensate for the surface charging effect. From these spectra, O/C ratios were calculated for the PEG brush and the galactose-modified PEG brush, which are 0.45 ± 0.01 and 0.48 ± 0.01, respectively. These values are in approximate agreement with those of theoretical ones, the O/C ratio is for 0.49 for the PEG brush (chemical formula $C_{47\sim49}O_{23\sim24}H_{94\sim98}NS$) and 0.53 for the galactose-modified PEG brush (chemical formula $C_{53\sim55}O_{28\sim29}H_{104\sim108}NS$). As can be seen from Figure S4, the slight decrease of O/C ratios seems plausible, as the O/C can be influenced by minor impurities at the surface, including carbon contamination of the gold surface due to environmental exposure after cleaning and other contaminants on the PEG or galactose-modified PEG brsuhes. Figure S5 shows the high-resolution spectra of C1s for the galactose-modified PEG brush, the PEG brush, and the bare gold surface. Spectrum of the bare gold surface (Figure S5 (a)) shows that there is no significant carbon oxidation. On the other hand, spectrum for the PEG brush is fitted with three peaks: hydrocarbon (C-H/C-C/C-S) at 284.6 ± 0.1 eV, hydroxyl/ether (C-O-X) at 286.4 ± 0.1 eV, and carbonyl (O=C-O(N)) at 289.3 eV (Figure S5 (b)). Compared with the former two surfaces, however, spectrum of the galactose-modified PEG brush surface can be fitted with four peaks: hydrocarbon (C-H/C-C/C-S) at 284.6 ± 0.1 eV, hydroxyl/ether (C-O-X) at 286.4 ± 0.1 eV, carbonyl (O=C-O(N)) at 289.3 ± 0.1 eV, and acetal (O-C-O) at 288 ± 0.1

eV(Figure S5 (b)). Since the O-C-O (288 ± 0.1) is unique to the carbohydrate acetal moiety, it can be included that the galactose has been introduced on the PEG brush by the enzymatic transglycosylation. Furthermore, Figure S6~S7 show the high-resolution spectra of O1s and Au 4f for the galactose-modified PEG brush surface. The O1s spectrum is fitted with three major species at 531.8 ± 0.1, 532.7 ± 0.1 and 533.4 ± 0.1 eV, which are attributed to carboxylate oxygen (O=C), alkoxyl (C-O), and acetal (C-O-C), respectively. The Au 4f spectrum is fitted with two major species at 83.9 ± 0.1 eV (Au $4f_{7/2}$) and 87.55 ± 0.1 eV (Au $4f_{5/2}$).

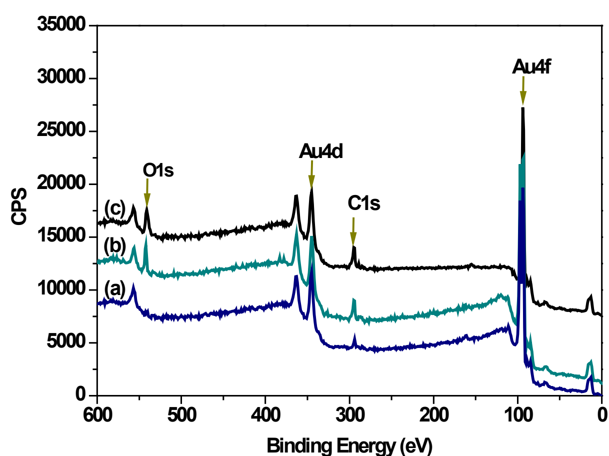
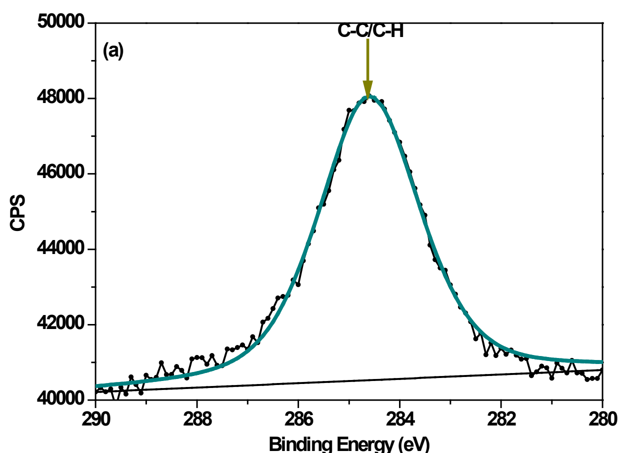


Fig. S4 Wide scan XPS spectra: (a) the bare gold surface, (b) the PEG brush surface, (c) the galactose-modified PEG brush surface.



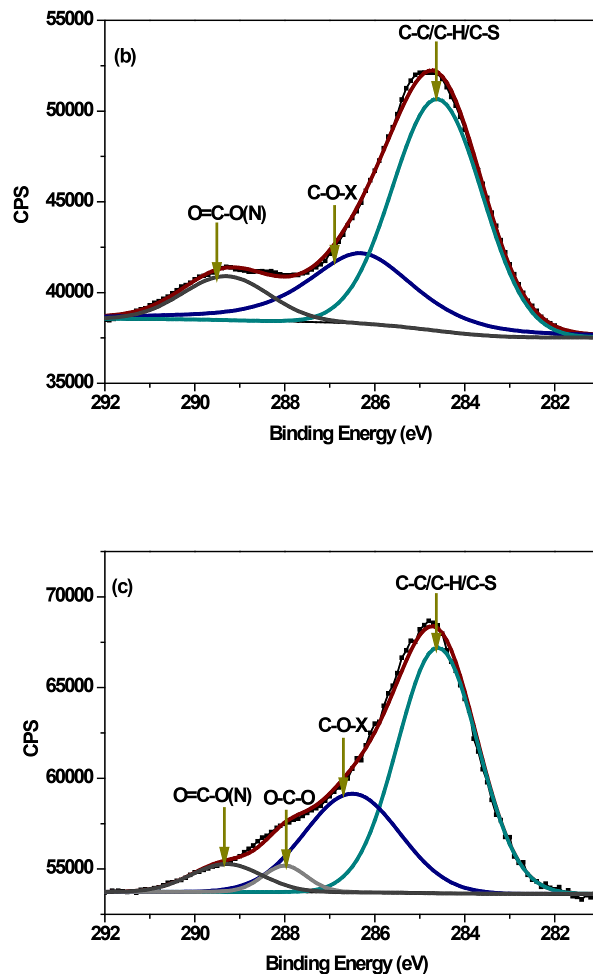


Fig. S5 XPS high-resolution C1s spectra: (a) the bare gold surface, (b) the PEG brush surface, (c) the galactose-modified PEG brush surface. (Peak area (%): (a) C-C/C-H: 100%; (b) C-C/C-H/C-S: 60.77%, C-O-X: 28.17%, C=O: 11.06%; (c) C-C/C-H/C-S: 61.20%, C-O-X: 28.94%, C=O: 6.16%, O-C-O: 3.7%.)

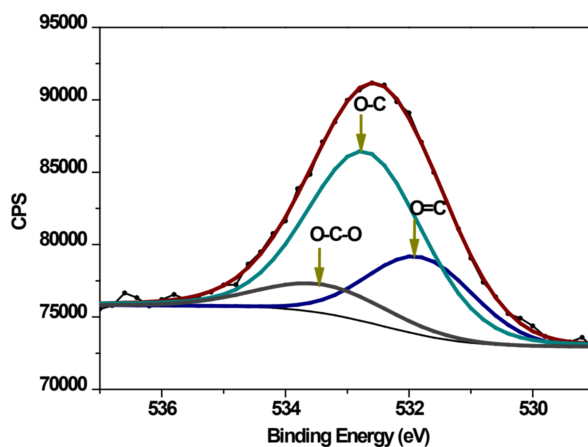


Fig. S6 XPS high-resolution O1s spectra of the galactose-modified PEG brush surface. (Peak area (%): O=C: 8.9%; O-C: 67.5%; O-C-O: 23.6%)

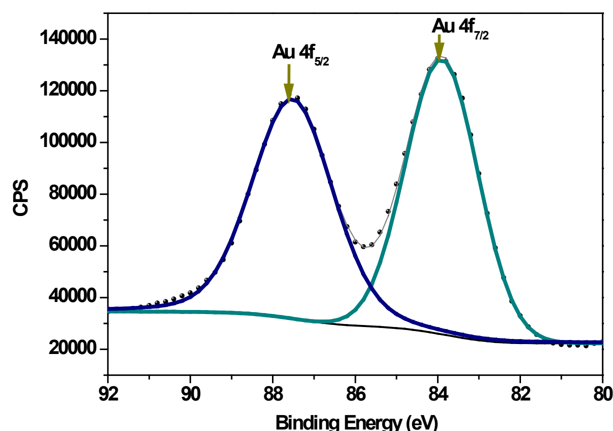
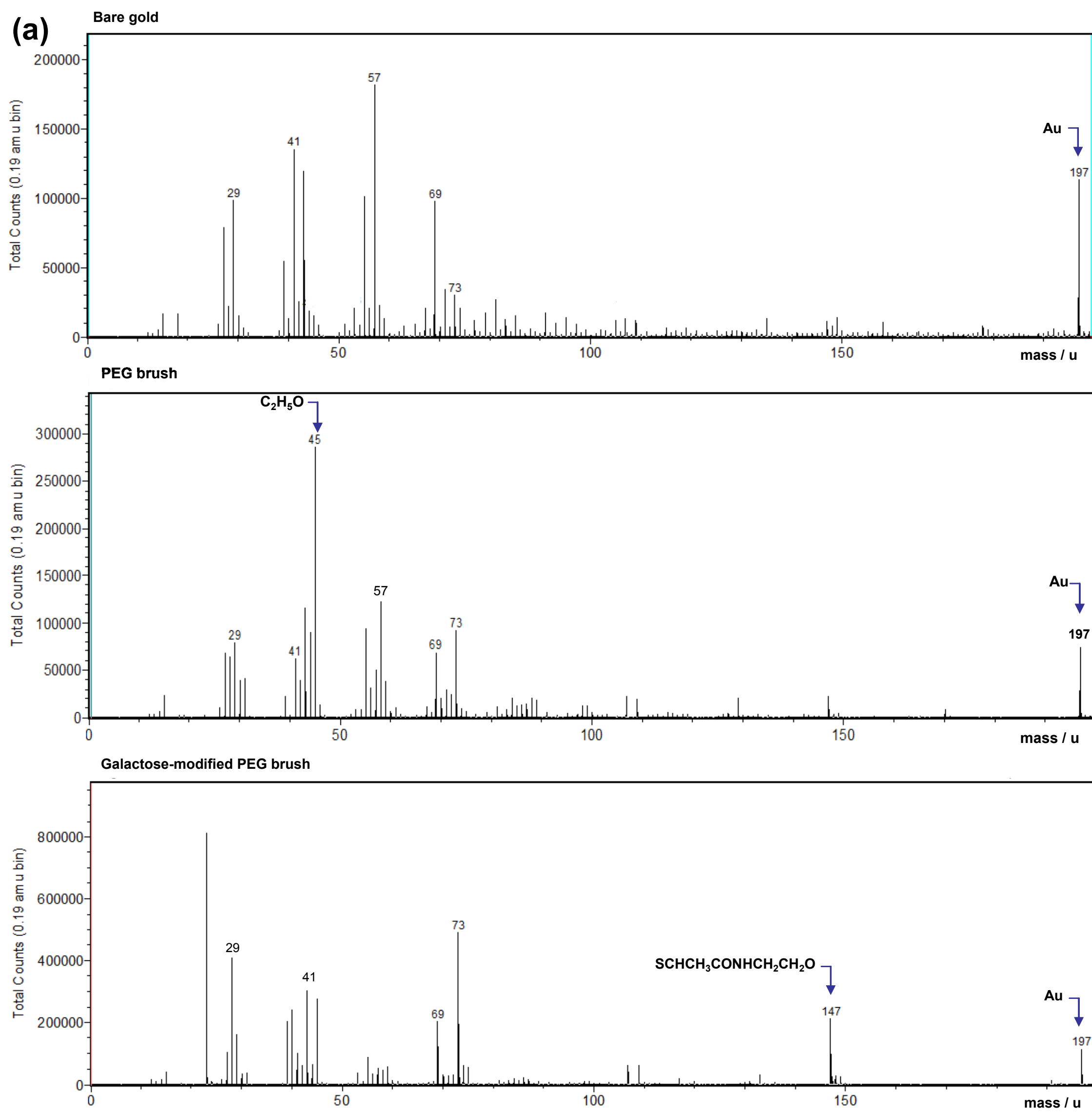


Fig. S7 XPS high-resolution Au 4f spectra of the galactose-modified PEG brush surface.

Analysis with Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). ToF-SIMS analysis was carried out with a TRIFT II time-of-flight secondary ion mass spectrometer (Physical Electronics, USA) equipped with a $^{69}Ga^+$ liquid-metal primary ion source. Primary ion bombardment was done by 15 keV Ga^+ ions with a pulsed current of 600 pA. A raster size of 100 × 100 µm was scanned and at least three different spots were analyzed. The total acquisition time was fixed to 180 s.

Results of TOF-SIMS on the galactose-modified PEG brush are presented in Figure S8 ((a)~(e)). We can be seen the carbohydrate-specific secondary fragment ions composed of carbon, hydrogen, and sulfur and oxygen. Fragment ions also exist as Au-S-C_xH_y, $AuSCH_2$, and $AuSC_2H_2$. These fragments are typically reported in TOF-SIMS spectra of alkanethiol SAMs on gold.[2] Additionally, the possible molecular ion M-H^+ ($C_{55}H_{108}O_{29}NS^+$) has been identified, thus providing further verification of a galactose moiety covalently bound to the PEG chain.


(a)
Bare gold
Total Counts (0.19 amu bin)
200000
150000
100000
50000
0
57
41
29
69
73
Au
197
0
50
100
150
mass / u
PEG brush
Total Counts (0.19 amu bin)
300000
250000
200000
150000
100000
50000
0
C2H5O
45
57
29
41
69
73
Au
197
0
50
100
150
mass / u
Galactose-modified PEG brush
Total Counts (0.19 amu bin)
800000
600000
400000
200000
0
29
41
69
73
SCHCH3CONHCH2CH2O
147
Au
197
0
50
100
150
mass / u

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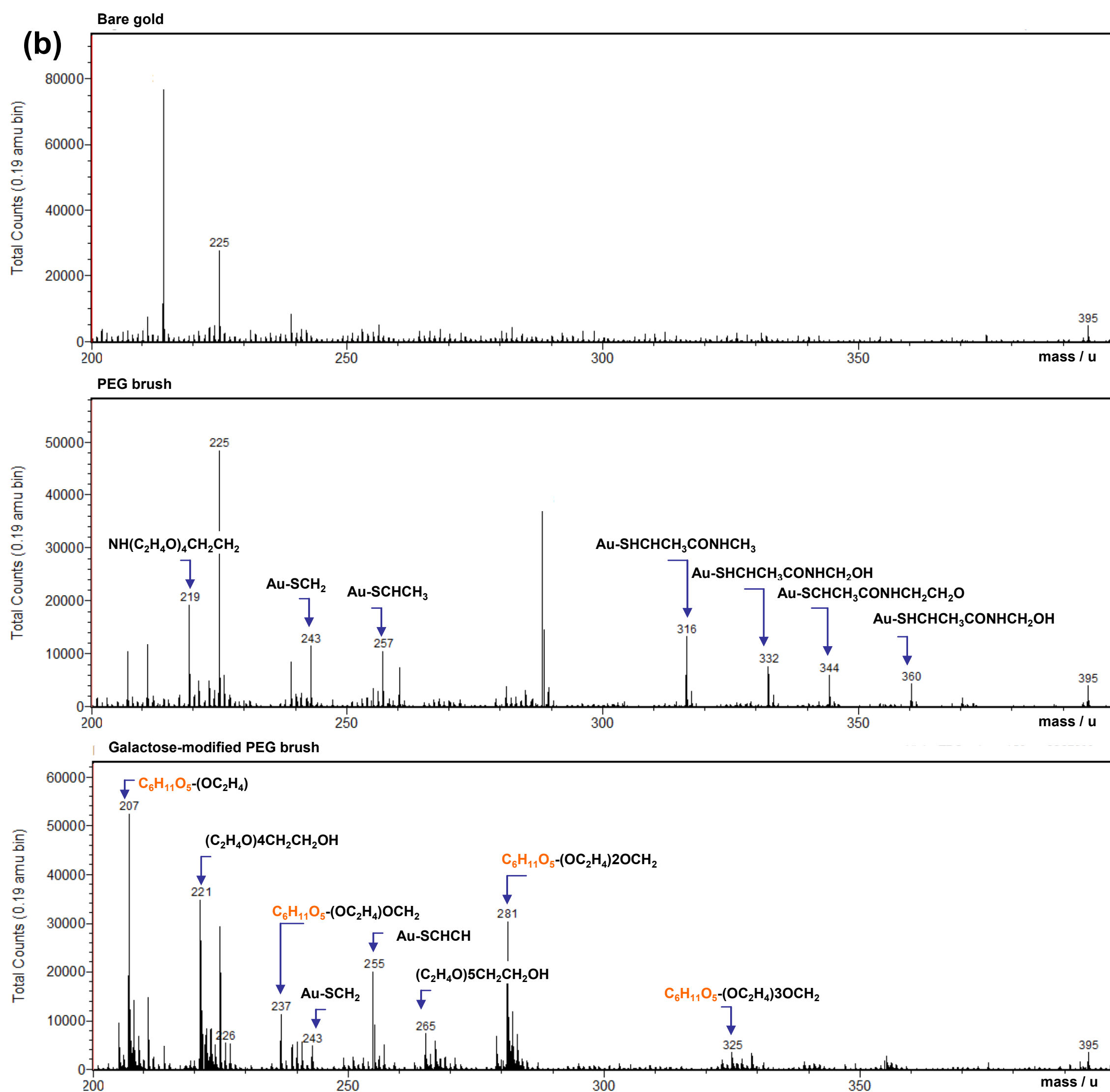

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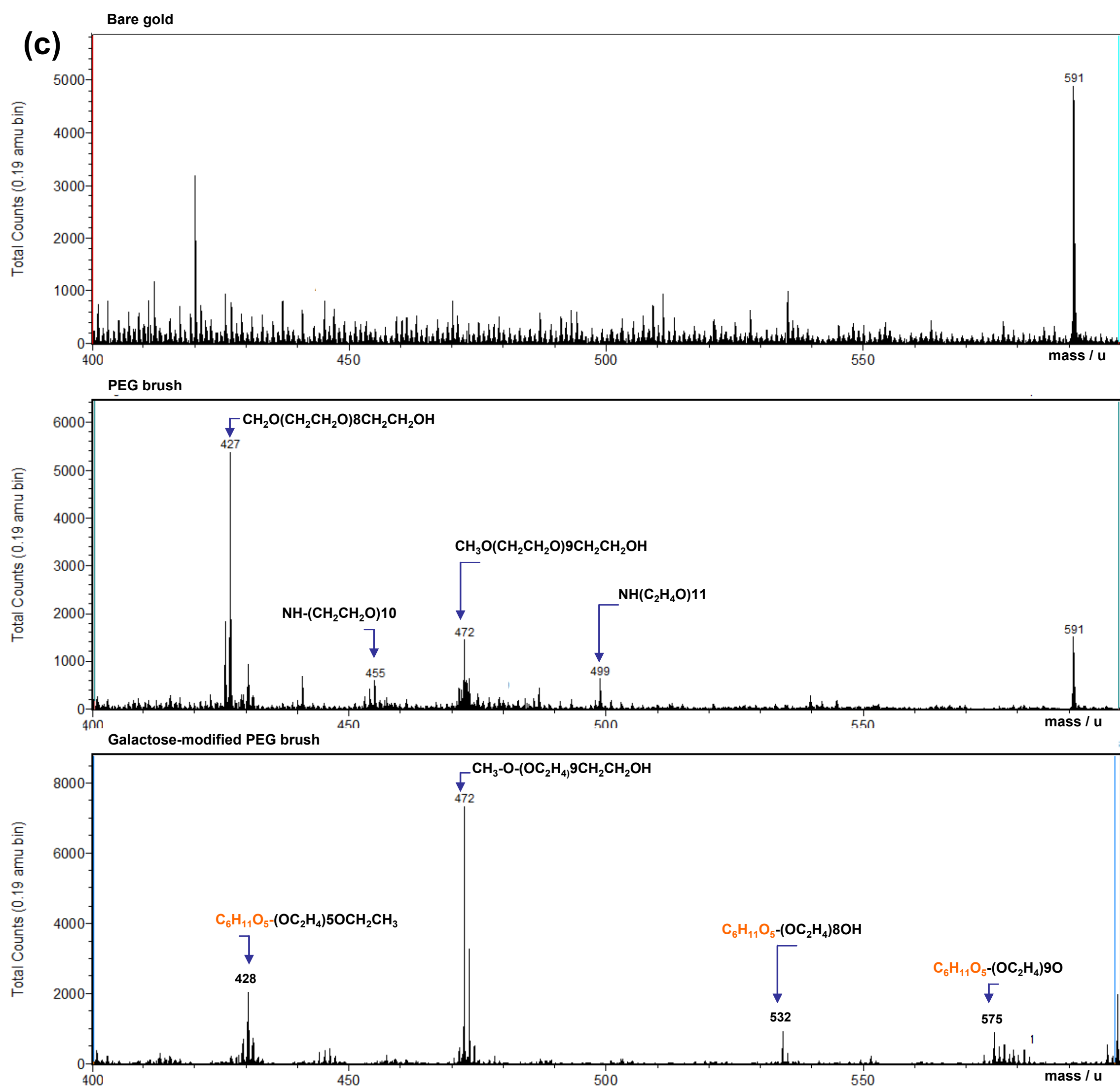

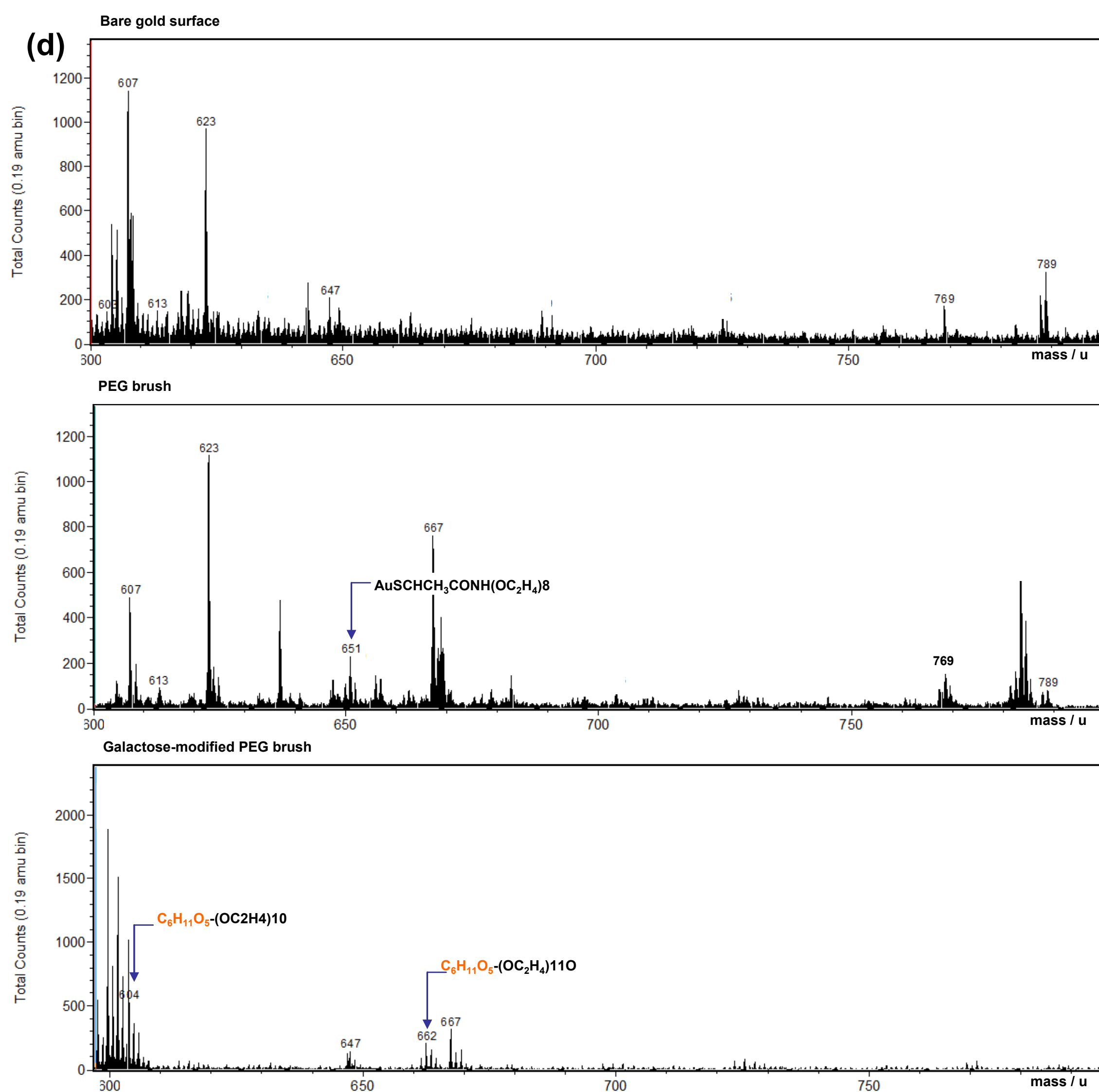
(d)
Bare gold surface
Total Counts (0.19 amu bin)
607
623
647
769
789
613
mass / u
PEG brush
AuSCHCH3CONH(OC2H4)8
667
651
769
789
613
607
Galactose-modified PEG brush
C6H11O5-(OC2H4)10
C6H11O5-(OC2H4)11O
604
647
662
667

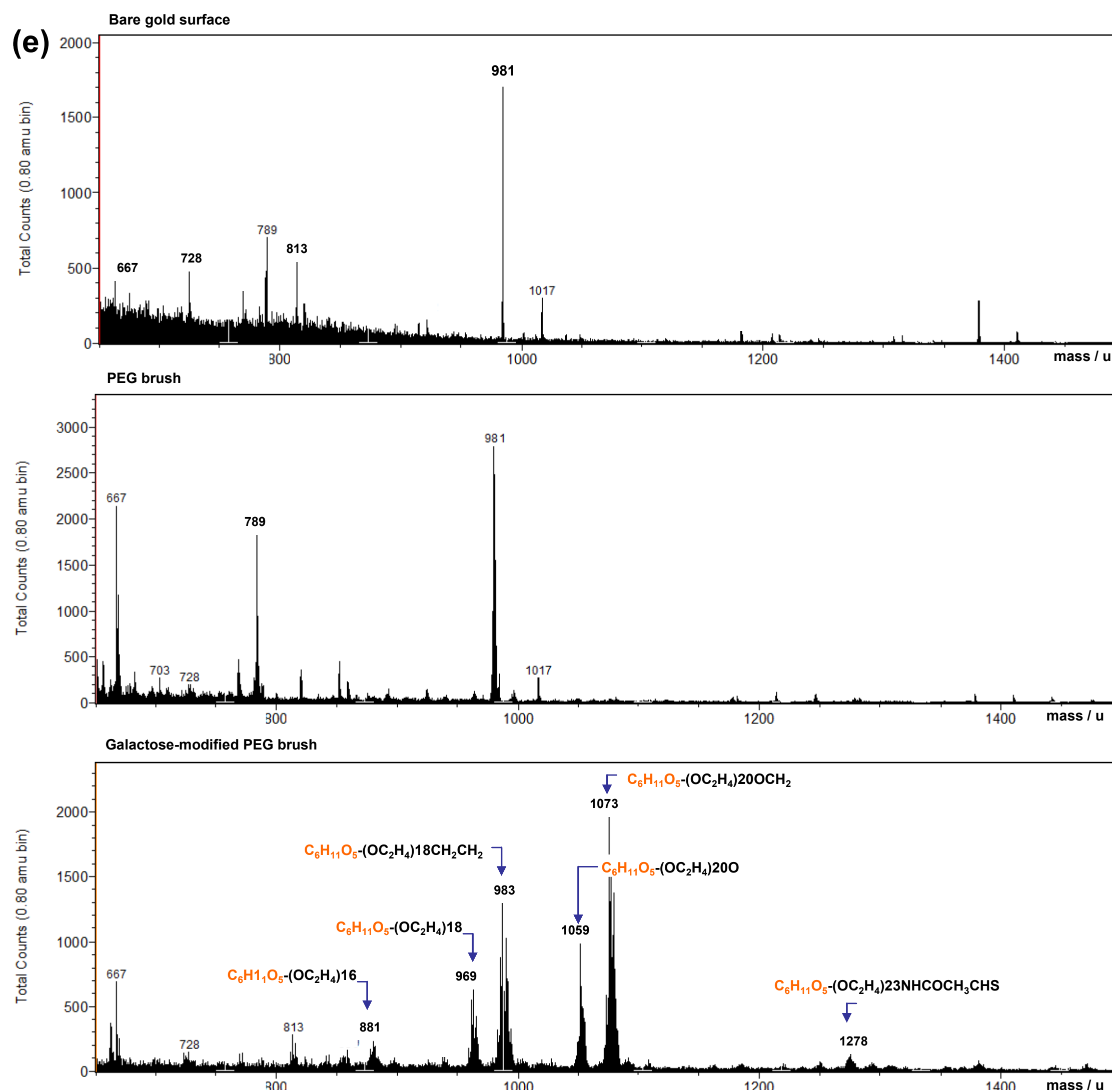

Fig. S8 Positive ToF-SIMS spectra of the bare gold surface, the PEG brush surface and the galactose-modified PEG brush surface. The mass regions: (a) 0 and 200 m/z, (b) 200 and 400 m/z, (c) 400 and 600 m/z, (d) 600 and 800 m/z, and (e) 800 and 1500 m/z. The color code (orange) from Scheme S1 was adopted to represent the galactose moiety.

Atomic Force Microscopy (AFM). AFM images of the PEG brush surfaces before a) and after b) enzymatic transglycosylation were obtained using a TM Lumina atomic force microscope (Nano IV, Veeco, USA), operated in tapping mode. Oxide sharpened SiN_3 cantilevers were used with a quoted spring constant of 0.04 N m^{-1}. Data were captured at a rate of 10 m s^{-1} in the z direction and a scan rate of 4 Hz. Fig. S9 shows that the blank PEG brushes have a peak-to-trough height of 5.25 nm, while this value changes slightly to

5.95 nm after the enzymatic transglycosylation. It indicates there are no bound enzymes on the galactose-modified PEG brush surface.

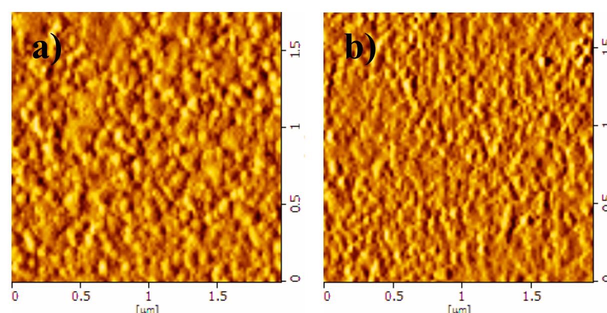

Fig. S9 AFM images of a) the PEG brush surface, and b) the galactose-modified PEG brush surface.

Fluorescence Microscopic Observation. The specific adsorption of FITC-RCA$_{120}$ on the galactose-modified PEG brushes was also confirmed by fluorescence microscopy. The sample was immersed in a PBS solution of FITC-RCA$_{120}$ (50.0 μg/mL) for a prescribed time at room temperature. It was then washed softly by immersing in PBS, and this procedure was repeated three times with fresh PBS. After that, the chip was dried under vacuum at room temperature. Fluorescence images were taken on an optical microscope (Eclipse TE2000, Nikon, Tokyo, Japan) equipped with a highly sensitive CCD camera (ORCA-ER, Hamamatsu Photonics, Shizuoka, Japan). The observation was made on at least five spots for each sample. Fig. S8 shows the typical fluorescence images of FITC-RCA$_{120}$ absorbed on the a) PEG brush surface and b) the galactose-modified PEG brush surface.

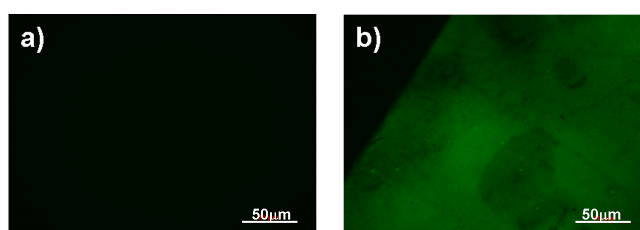

Fig. S10 Fluorescence images of FITC-RCA$_{120}$ absorbed on a) the PEG brush surface and b) the galactose-modified PEG brush surface.

Reference

1 Y. Okahata, T. Mori, H. Furusawa, T. Nohira, *Springer Ser. Chem. Sens. Biosens.*, 2007, **5**, 341.

2 Graham, D. J., Ratner, B. D., *Langmuir*, 2002, **18**, 5861.