

Supplementary Information for

Bifunctional cleavable probe for *in-situ* multiplexed glycan detection and imaging using mass spectrometry

Wen Ma,^a Shuting Xu,^a Honggang Nie,^b Bingyang Hu,^c Yu Bai,^{*a} and Huwei Liu^a

^a Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Institute of Analytical Chemistry, Peking University, Beijing 100871, P. R. China.

^b Analytical Instrumentation Center, Peking University, Beijing, 100871, P. R. China.

^c Institute of Hepatobiliary Surgery, Hospital of Hepatobiliary Surgery, Chinese People's Liberation Army General Hospital, Beijing 100853, P. R. China.

* Tel: +86 10 6275 8198; E-mail: yu.bai@pku.edu.cn

Contents

1. Chemicals and materials.....	S2
2. Apparatus.....	S2
3. Experimental section.....	S3
3.1 Synthesis of LCMPs.....	S3
3.2 <i>In-situ</i> analysis of cell surface glycans.....	S4
3.3 Monosaccharide inhibition.....	S5
3.4 Drug treatment.....	S5
3.5 Semi-quantitation of cell number.....	S5
3.6 Quantification of cell surface glycans.....	S5
3.7 MS imaging of tissue.....	S6
4. Supporting figures.....	S7
5. Supporting references.....	S11

1. Chemicals and materials

Concanavalin A (ConA) from *canavalia ensiformis*, *Sambucus nigra* agglutinin (SNA), fluorescein isothiocyanate conjugated concanavalin A (FITC-ConA), PEG NHS ester disulfide (4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-hexadeca-oxa-28,29 dithiahexapentacontanedioic acid di-*N*-succinimidyl ester, NHS-PEG-S-S-PEG-NHS, MW=1109.26), (11-mercaptopundecyl)hexa(ethylene glycol) HS-(CH₂)₁₁(OC₂CH₂)₆OH (mass tag1), [11-(methylcarbonylthio)undecyl]tetra(ethylene glycol) CH₃COS(CH₂)₁₁(OCH₂CH₂)₄OH, N-acetylneuraminic acid (sialic acid, Sia), methanol were purchased from Sigma-Aldrich. Pentaethylene glycol H(OCH₂CH₂)₅OH was from Aladdin Industrial Inc. Chloroauric acid hydrated (HAuCl₄·4H₂O), sodium borohydride, D-xylose (Xyl), sodium chloride (NaCl) were obtained from Sinopharm Chemical Reagent Co., Ltd. Sodium citrate, potassium carbonate, concentrated hydrochloric acid were from Xilong Chemical Co., Ltd. N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), N-Acetyl-D-glucosamine (GlcNAc), D-galactose (Gal), D-mannose (Man), D-fructose (Fru), tunicamycin (TM), 11-bromo-1-undecene, 2,5-dihydroxybenzoic acid (DHB) were from J&K Scientific Ltd. Dulbecco's modified Eagle's medium (DMEM), phosphate buffer saline (PBS) were from Hyclone, and fetal bovin serum (FBS) was from Life Technologies Co., Ltd. 4% Paraformaldehyde solution was purchased from Biotides Biotechnology Co., Ltd. Peptide-N-glycosidase (PNGase F) was from New England Biolabs (Ipswich, MA, USA). Benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside (BG) was gotten from Toronto Research Chemical Inc. (Canada). Water used for material synthesis and subsequent detection was from Wahaha Group Co., Ltd. Cell number was determined using a CELL-VU CBC counter from Advanced Mediatech International, Inc. (New York, USA). Wheat germ agglutinin (WGA), rhodamine conjugated wheat germ agglutinin (rhodamine-WGA), Cy3 labeled *Sambucus nigra* lectin (Cy3-SNA) was from Vector Laboratories.

2. Apparatus

MALDI-TOF MS spectra were obtained by a Bruker Daltonics ultraflex TOF mass spectrometer in reflection or linear mode. The laser frequency was set at 20 Hz and laser intensity was set to be 47%. Step by step synthesis of LCMPs were verified by MALDI-TOF MS, the successful modification of ConA lectin was confirmed at *m/z* ranging from 1 kDa to 120 kDa in linear mode, using DHB as matrix while functionalization of MTs was confirmed at *m/z* ranging from 700 Da to 1000 Da in reflection mode without additional matrix. Ultraviolet and visible (UV-Vis) spectra were measured using UV-Vis spectrophotometer (Perkin Elmer, Lambda 35) at wavenumber from 400 nm to 700 nm. For transmission electron microscopy (TEM) characterization, LCMPs were collected on carbon-coated copper grids. TEM images were recorded on a JEOL JEM-2100F at 200kV. Confocal images of different probes such as Au NPs, Au-MT1 and Au@ConA(FITC)-MT1 (or Au NPs, Au-MT2, Au@SNA(Cy3)-MT2 and Au NPs,

Au-MT3, Au@WGA(Rhodamine)-MT3) were collected by fluorescence microscope (Nikon, Eclipse Ti-U), probe solutions were dropped onto a glass slide. After drying, fluorescence released from probes was measured under excitation. MS imaging of tissues was conducted by Shimadzu iMScope TRIO Imaging Mass Microscope. Sample voltage and detection voltage were set to be 3.5 kV and 1.8 kV, respectively. Number of shots was 200, repetition rate was 1000 Hz, laser diameter was set to be 25 μm and intensity was 32. $^1\text{H-NMR}$ spectrum was recorded on a Bruker ARX400 FT-NMR spectrometer (at 400 MHz). Precise mass measurements of MT1, MT2, MT3 and three characteristic peaks of LCMP1 (as an example) were conducted by Thermo Q-Exactive mass spectrometer (Thermo, San Jose, CA) equipped with ESI ion source and FT-ICR MS equipped with MALDI ion source (Solarix XR, Bruker Daltonics).

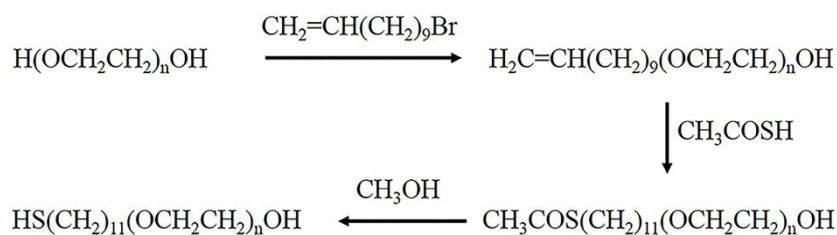
3. Experimental section

3.1 Synthesis of LCMPs

Preparation of MT2 and MT3

MT1 (11-mercaptoundecyl)hexa(ethylene glycol) $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OH}$ was from Sigma-Aldrich. MT2 (11-mercaptoundecyl)tetra(ethylene glycol) $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_4\text{OH}$ was synthesized from [11-(methylcarbonylthio)undecyl]tetra(ethylene glycol) $\text{CH}_3\text{COS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_4\text{OH}$ through a simple alcoholysis reaction. In detail, 0.236 mmol [11-(methylcarbonylthio)undecyl]tetra(ethylene glycol) was dissolved in 20 mL methanol, then 1 mL concentrated hydrochloric acid was added and the mixture was refluxed for 12 h. MT3 (11-mercaptoundecyl)penta(ethylene glycol) $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_5\text{OH}$ was synthesized from pentaethylene glycol $\text{H}(\text{OCH}_2\text{CH}_2)_5\text{OH}$ according to a generic synthetic route^{S1} shown in Scheme 1 and each step was purified by column chromatography. All the final products were confirmed by high resolution MS and $^1\text{H-NMR}$. ESI-MS (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{19}\text{H}_{40}\text{O}_5\text{SNa}$ (MT2), 403.2488, found 403.2486, $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{21}\text{H}_{44}\text{O}_6\text{SNa}$ (MT3), 447.2750, found 447.2745, $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{23}\text{H}_{48}\text{O}_7\text{SNa}$ (MT1), 491.3013, found 491.3011. $^1\text{H NMR}$ (400 MHz, CDCl_3) for MT2: 3.74-3.57 (m, 16H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), 3.46-3.43 (t, 2H, $-\text{CH}_2-\text{CH}_2-\text{O}-$), 2.55-2.49 (q, 2H, $-\text{CH}_2-\text{SH}$), 1.64-1.54 (m, 4H, alkyl chain), 1.39-1.27 (m, 14H, alkyl chain). $^1\text{H NMR}$ (400 MHz, CDCl_3) for MT3: 3.66-3.57 (m, 20H, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{O}-$), 3.46-3.43 (t, 2H, $-\text{CH}_2-\text{CH}_2-\text{O}-$), 2.55-2.49 (q, 2H, $-\text{CH}_2-\text{SH}$), 1.62-1.54 (m, 4H, alkyl chain), 1.39-1.27 (m, 14H, alkyl chain). FT-ICR MS of LCMP1 three characteristic peaks: calcd for $\text{C}_{46}\text{H}_{94}\text{O}_{14}\text{Na}$ ($\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_6-(\text{CH}_2)_{11}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6-\text{OH}+\text{Na}$) 893.6536, found 893.6506, calcd for $\text{C}_{46}\text{H}_{94}\text{O}_{14}\text{SNa}$ ($\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_6-(\text{CH}_2)_{11}-\text{S}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6-\text{OH}+\text{Na}$) 925.6257, found 925.6225, calcd for $\text{C}_{46}\text{H}_{94}\text{O}_{14}\text{S}_2\text{Na}$ ($\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_6-(\text{CH}_2)_{11}-\text{S}-\text{S}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6-\text{OH}+\text{Na}$) 957.5977, found 957.5944.

Scheme S1 Preparation of MTs from a generic route



Synthesis of Au NPs

Au NPs were synthesized through a common protocol.^{S2} Briefly, 100 mL 1 mM HAuCl₄ in water was brought to boil followed by adding 10 mL 38.8 mM sodium citrate, boiling was continued for additional 10 min. Then, heating source was removed and reaction was conducted continuously for another 15 min. Size of Au NPs was estimated using Haiss *et al.* method^{S3} by UV-Vis spectroscopy. Based on this method, diameter of Au NPs was about 13 nm with concentration of 10 nM.

Synthesis of LCMPs

LCMPs were prepared through a simple and generic method^{S4} with further modification. In detail, different lectins (ConA, SNA and WGA) were reacted with a PEG linker NHS-PEG-S-S-PEG-NHS at 2:1 molar ratio in 10 mM HEPES solution (10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, 137 mM NaCl, pH 7.5) for 12 h, followed by adding 10 nM Au NPs and K₂CO₃ (final concentration at 1.8 mM) into the mixture and further reacted for 4 h. After then, different MTs were added into solution, respectively. The obtained functional LCMPs were washed by water for at least three times and dispersed in deionized water, stored at 4 °C until use. For reaction condition optimization, different amount of lectins (Au:ConA=1:50, 100, 300, 500, 800, 1000) and MT1 (Au:MT1=1:1000, 5000, 10000, 50000) were tested to get the best formulation (Au: ConA: MT1=1: 100: 5000). It was found that when lectin ratio larger than 100, signal intensity would decrease, this may due to lectin fully occupied the active site of Au surface that inhibited the subsequent functionalization of MT1 on Au surface. Besides, signal intensity almost remained the same after 5000 fold regardless of the ever-increasing amount of MT1. Similar results were also observed for SNA and WGA lectins, so we fixed the optimum formula to be Au: Lectin: MTs=1: 100: 5000.

3.2 *In-situ* analysis of cell surface glycans

MCF-7 cells were cultured in DMEM with 10% FBS at 37 °C in an atmosphere of 5% CO₂. For *in-situ* analysis of cell surface glycans, conductive ITO glass from Bruker Daltonics (glass slides for MALDI imaging, Part No.: 8237001) was used for culturing living MCF-7 cells by directly seeded cell suspension

on ITO surface at concentration of 10^5 cells/mL. After 24 h incubation, cells were grown on ITO surface and further fixed by paraformaldehyde solution (4%) for 10 min and washed by PBS buffer for three times. LCMPs (200 μ L, 1 nM) in PBS buffer containing 0.1 mM Ca^{2+} and Mn^{2+} were added to the surface of ITO glass and reacted with cell surface glycans for one hour under 37 °C. Then, cells were further washed by PBS buffer (at least three times, 1 mL each time) to fully remove non-specific adsorption. After natural drying, cells on ITO glass were directly detected by LDI-TOF MS without additional matrix. For multiplexed analysis, mixture of LCMP1, LCMP2 and LCMP3 (200 μ L, 1 nM for each probe) was used as labelling probes and following the same procedure.

3.3 Monosaccharide inhibition

Each LCMP (10 nM) was preincubated with 100 μ M monosaccharides (mannose, fructose, xylose, galactose, sialic acid and N-acetylglucosamine) respectively for 2 h at room temperature, preinhibited probes were purified by washing and separated by centrifugation at 10500 rpm for 10 min, then dispersed in PBS buffer containing 0.1 mM Ca^{2+} and Mn^{2+} . Cells (10^5 cells/mL) on ITO glass surface were incubated with preinhibited LCMPs (200 μ L 1 nM) following the above procedure, and directly detected by LDI-MS at the same instrument condition.

3.4 Drug treatment

For evaluation of cell surface glycans alteration under different drug stimulation, mannose on cell surface was used as an example. Cells at density of 10^5 cells/mL were incubated in the culture medium containing 12 μ M N-glycan inhibitor TM or 120 μ M O-glycan inhibitor BG for 24 h. Enzymatic cleavage of N-glycans was conducted by adding 3000 units PNGase F to cell suspension and then mildly shaking for 24 h to remove N-glycans. Besides, cells treated by 12 μ M TM or 120 μ M BG also underwent enzymatic digestion according to the same procedure.

3.5 Semi-quantitation of cells

To get certain amount of cell number, cells were trypsinized to form cell suspension at different concentration. Mixture containing 100 μ L cell suspension and 30 μ L LCMP1 (1nM) were incubated in a tube for one hour, after washed by PBS for at least three times, cell precipitation was collected through centrifugation. Cell precipitation labeled by LCMP1 was mixed with certain amount of internal standard Au-MT2 and the mixture was directly deposited on MALDI plate for further detection. Relative intensity (RI) of LCMP1/Au-MT2 showed linear correlation with cell concentration. Limits of detection of cell was determined according to signal noise ratio (S/N larger than 3).

3.6 Quantification of cell surface glycans

Monosaccharide competition strategy was used for quantification of cell surface glycans. Correlation between relative intensity (LCMP1/Au-MT2, LCMP2/Au-MT1, LCMP3/Au-MT1) and cell number was measured following the above method. If we defined x is the amount of glycan (mannose, sialic acid or N-acetylglucosamine) on cell surface and y is signal intensity that each glycan produced, for each LCMP, the RI was proportional to cell number (n_{cell}) in the range of 1000-10000 when we fixed probe amount

$$\begin{aligned} \text{RI} &= x n_{\text{cell}} y + b_1 \\ &= k_1 n_{\text{cell}} + b'_1 \end{aligned}$$

On the other hand, free monosacchrides (a) were added for competitive binding with LCMPs, resulting in loss of RI. Curve between RI and (a) was found linear correlation between 20 nM-100 nM (absolute amount 2 pmol-10 pmol) when we fixing cell number and probe amount

$$\begin{aligned} \text{RI} &= (x n_{\text{cell}} - a) y + b_2 \\ &= k_2 a + b'_2 \end{aligned}$$

Assuming that the mannose had the same binding kinetics as that on MCF-7 cell surface, the average amount of glycan (x) on MCF-7 cell surface could be calculated by the slopes (k_1 and k_2) of these two curves.

3.7 MS imaging of tissue

Human cancerous and paracancerous tissues were collected immediately after surgical resection at the Chinese PLA General Hospital. All the investigation protocols in this study were approved by institutional review board of the Chinese PLA General Hospital and institutional ethics committees of Peking University with informed consent from patients. Tissues were embedded by 15% gelatin solution and was cut to 8 μm slice under $-20\text{ }^\circ\text{C}$ with serial sections. One specimen was used for HE staining and the other serial section was used for MS imaging. To eliminate systematic interference, a couple of cancer and paracancerous tissues was placed on the same ITO glass slide, certain amount of LCMPs (200 μL , 5 nM) were added to the surface of tissues and incubated at $37\text{ }^\circ\text{C}$ for an hour. Then, tissues were gently washed by PBS buffer for three times (1 mL each time) and finally washed by deionized water to remove all unbounded LCMPs. After washing and drying, tissues were directly detected by imaging MS to get intrinsic distribution pattern of different glycans (laser diameter 25 μm , pitch: 30, 30 μm , pixels: 10000 (100, 100)). Following MS analysis, data was loaded into Imaging MS solution software to generate overall average mass spectra. In total, three pairs of liver tissues and two pairs of colorectal tissues were tested, at least three repetitive experiments were conducted for each pair to get the solid results.

4 Supporting figures

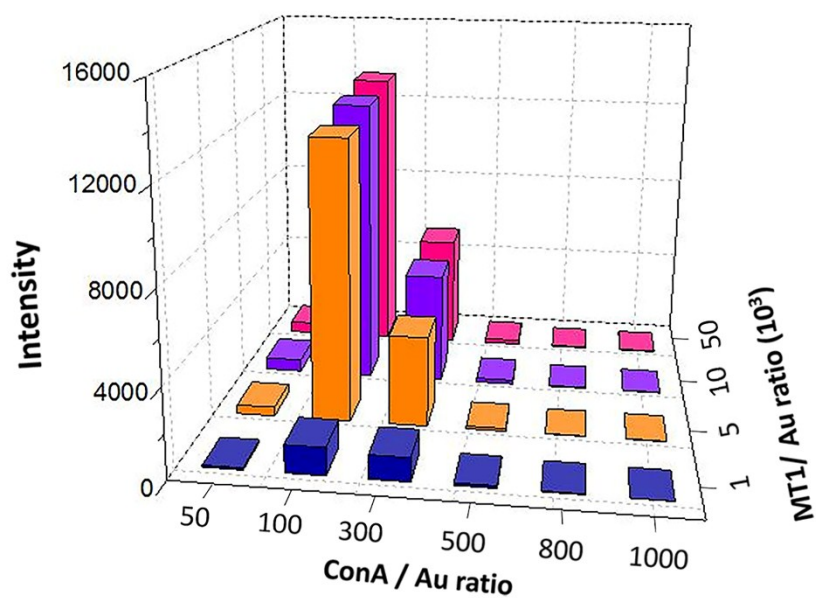


Figure S1. Reaction condition optimization

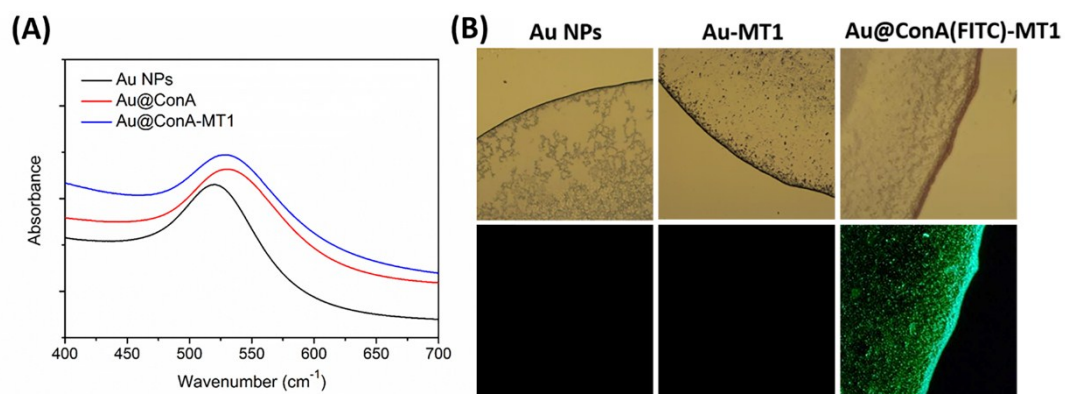


Figure S2 (A) UV-Vis spectra of Au NPs, Au@ConA, Au@ConA-MT1 and (B) Bright field image and confocal image of Au NPs, Au-MT1 and Au@ ConA(FITC)-MT1

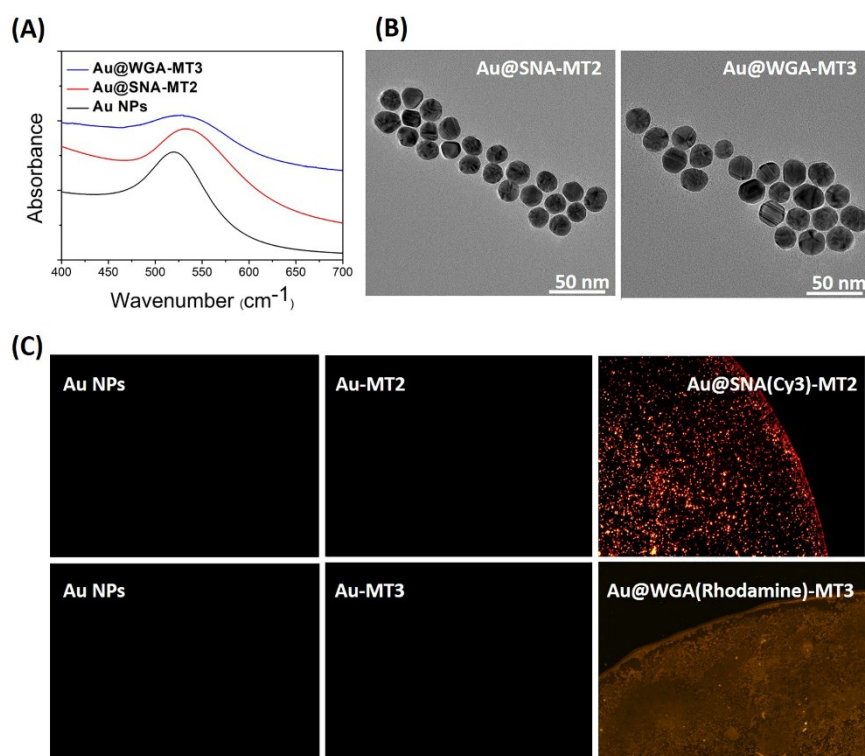


Figure S3 (A) UV-Vis spectra of Au NPs, Au@SNA-MT2 and Au@WGA-MT3 (B) TEM image of Au@SNA-MT2 and Au@WGA-MT3, (C) Confocal image of Au NPs, Au-MT2, Au-MT3, Au@SNA(Cy3)-MT2 and Au@WGA(Rhodamine)-MT3.

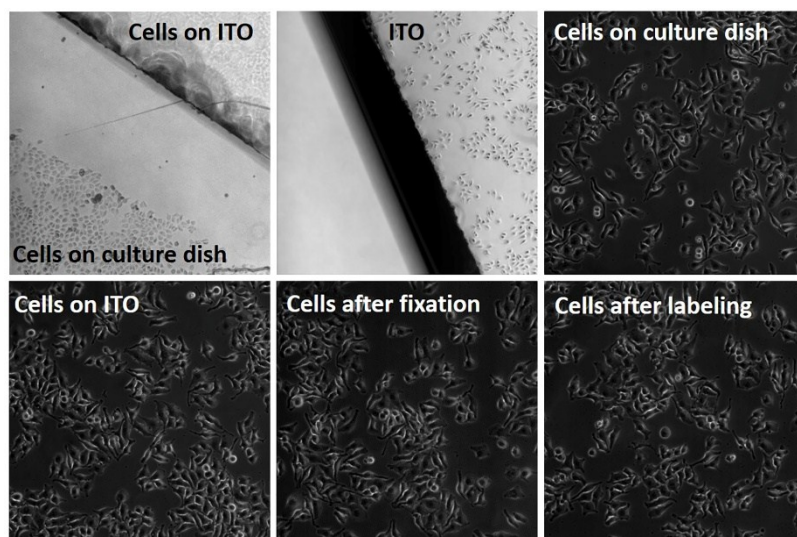


Figure S4. Cells grew on culture dish, ITO glass surface and living status after fixation and labeled by different LCMPs

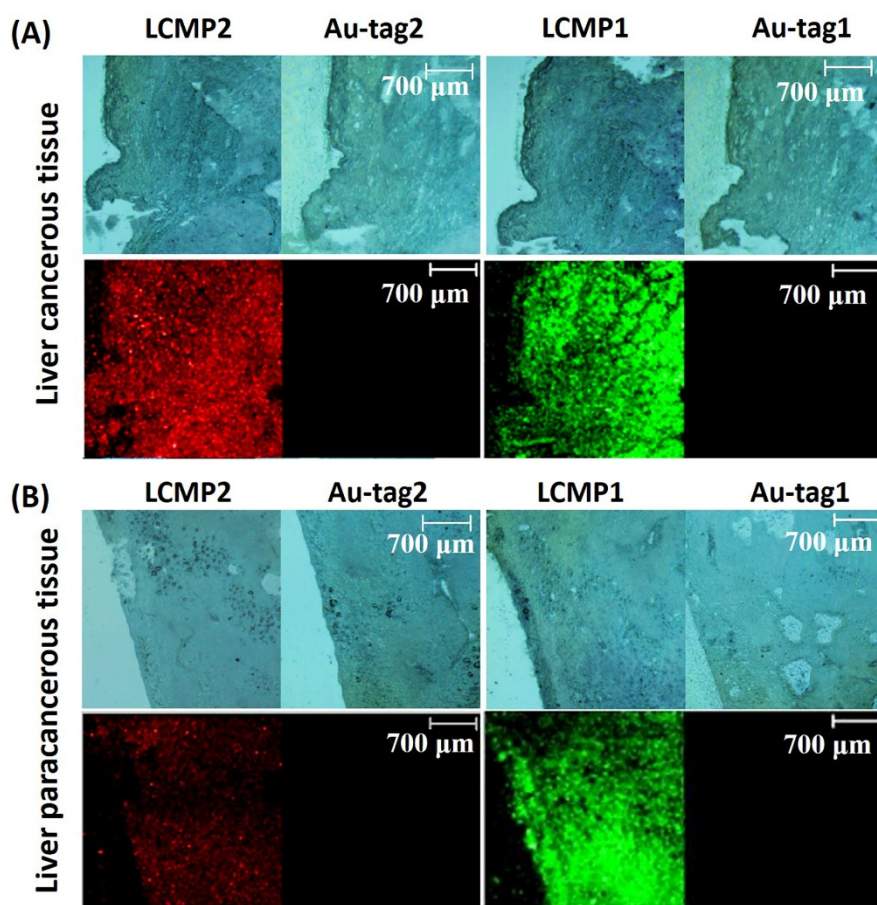


Figure S5 Mass imaging spectra of different probes LCMP2, Au-tag2, LCMP1, Au-tag1 on (A) liver cancerous tissue and (B) liver paracancerous tissue. LCMP1 and LCMP2 reflected expression of mannose and sialic acid glycans on tissues respectively.

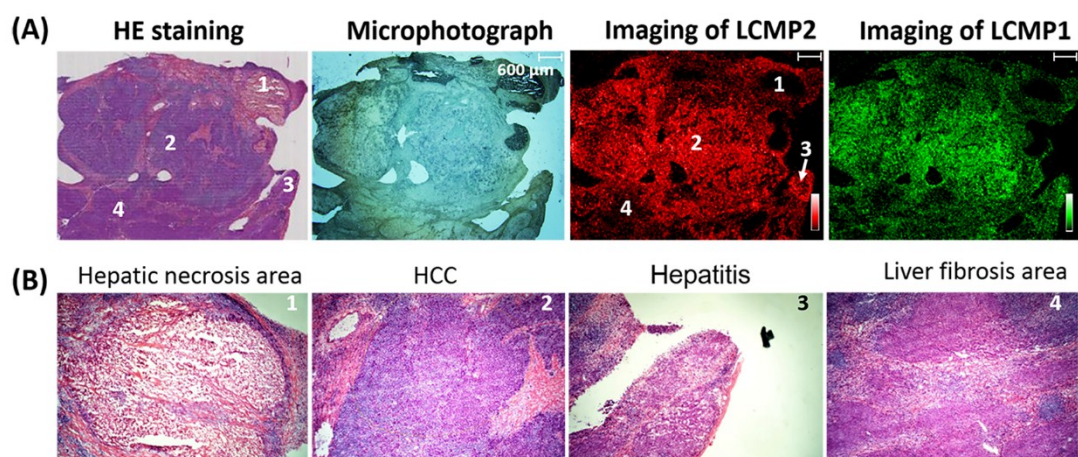


Figure S6 (A) HE staining, microphotograph, MS imaging of LCMP2 and LCMP1 on liver tissue from a HCC patient. (B) Enlarged image of four selected areas in HE staining image, representing hepatic necrosis area, HCC area, hepatitis area and liver fibrosis area, respectively.

5 Supporting references

- S1 C. Pale-Grosdemange, E. S. Simon, K. L. Prime and G. M. Whitesides, *J. Am. Chem. Soc.*, 1991, **113**, 12-20.
- S2 K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, *Anal. Chem.*, 1995, **67**, 735-743.
- S3 W. Haiss, N. T. K. Thanh, J. Aveyard and D. G. Fernig, *Anal. Chem.*, 2007, **79**, 4215-4221.
- S4 L. R. Hirsch, J. B. Jackson, A. Lee, N. J. Halas and J. West, *Anal. Chem.*, 2003, **75**, 2377-2381.