Influenza-binding sialylated polymer coated gold nanoparticles prepared via RAFT polymerization and reductive amination

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EXPERIMENTAL SECTION

Materials

N-(2-Hydroxypropyl)methacrylamide (HPMA) and N-(3-aminopropyl)methacrylamide (APMA) were purchased from Polysciences. α-2,6-Sialylactose was purchased from Carbosynth. All other chemicals were obtained from Sigma-Aldrich. Milli-Q grade water was used for all experiments.

RAFT copolymerization of HPMA and APMA

Four types of copolymers of HPMA and APMA with different degrees of polymerization and APMA contents were synthesized by aqueous RAFT polymerization as previously reported. In brief, CTP (4-cyanopentanoic acid dithiobenzoate, 97%) and ACVA (4,4'-azobis-4-cyanopentanoic acid, >98%) were weighed with molar ratio of 2:1 and added to a round-bottomed flask equipped with a stir bar. HPMA and APMA with two proportions of 3:1 and 9:1 ([M]: [CTA] = 100:1 and 25:1) were then added to the flask and dissolved in a mixture of water and dioxane (2:1 in volume). After complete dissolution of monomer and CTP, the final solution was degassed by nitrogen bubbling for 30 min. The round bottomed flask was then placed in a preheated oil bath at 70°C. After reaction for overnight, the reaction was quenched by cooling the reaction vessel in an ice bath followed by exposing to air. The copolymer was obtained as pink powder after precipitation in pre-cooled acetone and drying under vacuum.

Synthesis of glycopolymers

α-2,6-sialyllactose was conjugated to the primary amino group of poly(HPMA-co-APMA) by reductive amination using NaBH(OAc)₃ as a reducing agent. The NaBH(OAc)₃ was added to the mixture of polymer and α-2,6-sialyllactose in 2 mL of DMSO. The reaction mixture was incubated at 50 ºC for 4 days while it was constantly stirred, followed by dialysis against distilled water for 3 days using a Spectra membrane (molecular weight cutoff of 8000) to remove unreacted saccharide. Then the polymer was recovered in dry state by freeze-drying. Lactose modified polymers were synthesized in the same way, except using lactose instead of α-2,6-sialyllactose as reagent.
Physicochemical characterization of glycopolymers

**1H-NMR spectroscopy.** 1H-NMR spectra were recorded on a Bruker Avance DRX 300 MHz spectrophotometer. Chemical shifts are reported relative to tetramethylsilane (TMS).

**Size exclusion chromatography (SEC).** For analytical SEC measurements in hexafluoroisopropanol (HFIP) (containing 3.0 g/L of potassium trifluoroacetate), a PU 2080+ pump, an auto sampler AS1555, a UV-detector UV 1575 (detection at 230 nm) and an RI-detector RI2080+ from JASCO were used. Columns packed with modified silica were obtained from MZ-Analysentechnik: PFG columns, particle size 7 µm, porosity 100 Å and 1000 Å. Calibration was carried out with poly(methyl methacrylate) standards purchased from PSS.

**Ninhydrin assay.** The ratio of substitution of primary amino group in polymers with saccharide was determined by the ninhydrin assay. Solutions of the glycopolymers were prepared in 0.1 M NaHCO₃ buffer. Ninhydrin was added and tubes were placed in a boiling water bath for 10mins. The solutions were cooled and the absorbance value at 570 nm was recorded. P(HPMA-co-APMA) copolymer solution were used to generate a standard curve.

**Preparation of glycopolymer decorated goldNP**

Citrate stabilized gold nanoparticle was synthesized via classic Turkevich and Frens method by direct reduction of gold salt in the presence of sodium citrate as reducing agent under reflux. Briefly, 24.4 mg of HAuCl₄ was dissolved in 50 mL of milliQ water in a round bottom flask pre-cleaned with aqua regia solution (mixture of HCl and HNO₃ at a ratio of 3:1) and heated to reflux. Then, 5ml of a sodium citrate stock solution with a concentration of 10 mg/mL was added to the gold salt solution and kept stirring for another 5 min under reflux. The obtained red colored solution depicted the formation of gold nanoparticles. After cooling to room temperature, the colloidal gold can be stored in fridge stably for a couple of years.

(2,6-Sialyllactose-polymer decorated gold nanoparticles were prepared via “grafting-to” strategy by treating citrate stabilized goldNP with an aqueous solution of (2,6-sialyllactose conjugated glycopolymers. Briefly, 8 mg of (2,6-sialyllactose-polymer were dissolved in 200 µL of milliQ water and then added into 8 mL of citrate stabilized goldNP under constant stirring at
room temperature for overnight. The glycoconjugate was purified using centrifugation at speed of 15000 g and washed with milliQ water for 3 times. After redispersion in water, \(-2,6\)-sialyllactose-polymer decorated goldNP were stored in fridge prior to usage. Lactose-polymer modified goldNP were obtained via exchange of citrate stabilized goldNP with lactose functionalized polymers in the same way.

**Physicochemical characterization of glycopolymer decorated goldNP**

DLS (Dynamic light scattering). DLS was performed at 25°C using a scattering angle of 173° with a Malvern Zetasizer Nanos S instrument equipped with a 4 mW He-Ne laser operating at 633 nm, an avalanche photodiode detector with high quantum efficiency, and an ALV-LSE-5003 multiple digital correlator electronics system. The intensity-average diameter and polydispersity of the goldNP and viral particles were calculated by cumulants analysis of the experimental correlation function using Dispersion Technology software version 7.20.

UV-VIS (Ultraviolet-Visible spectroscopy). The UV-VIS spectra were obtained with a Shimadzu UV-1650PC spectrophotometer. The mixture of different glycopolymer coated gold nanoparticles with two types of lectins were placed in plastic cuvettes, and spectra were recorded in the 350-800 nm wavelength range at room temperature.

TEM (Transmission electron microscopy). TEM images were recorded on a Jeol 1010 transmission electron microscope. GoldNP were incubated with formalin inactivated PR8 influenza virus for overnight at 4°C prior to TEM measurement and placed onto an electron microscopical grids followed by negative staining.

TGA (Thermogravimetric analysis). The grafting efficiency on the GoldNP was determined by thermogravimetric analysis using a TGA Q 5000 from TA Instruments in a modulated mode, with a heating linear ramp of 20 °C/min from room temperature to 1000 °C and under N\(_2\) atmosphere. The graft density of polymer chains could be calculated as follows:

\[
\text{Surface density of polymer (chains/nm}^2) = \frac{W_{\text{polymer}}}{\rho \times V_{\text{particle}} \times NA \times M_{\text{polymer}}}
\]

Where, \(W_{\text{polymer}}\) is the percent weight loss corresponding to the decomposition of polymer, \(\rho\) is the density of gold (19.32 g/cm\(^3\)), \(V_{\text{particle}}\) is the volume of one goldNP calculated from the size measured by TEM (2572 nm\(^3\)), \(NA\) is Avogadro constant (6.02×10\(^{23}\) mol\(^{-1}\)), \(M_{\text{polymer}}\) is the
molecular weight of polymer (42716 g/mol), and $S_{\text{particle}}$ is the surface area of one goldNP (940 nm$^2$).  

**Binding assay of glycopolymer decorated goldNP**

To investigate the binding properties of glycopolymer decorated goldNP with lectins, 10 µL of ω-2,6-sialyllactose-polymer decorated goldNP (2.5 mg/mL in gold content) was diluted in 950 µL of PBS solution prior to the addition of 40 µL of SNA stock solution in PBS with concentration of 1 mg/ml and incubated at 4°C for overnight with lectin. MAA was used as a control for binding to the goldNP. The change in size of the goldNP was determined via DLS measurements. The surface plasmon resonance peak of goldNP before and after incubation with lectin was obtained via UV-VIS spectroscopy. To investigate the specific lectin-binding property of ω-2,6-sialyllactose-polymer decorated goldNPs, lactose-polymer decorated goldNPs were also treated with lectins as control.

To investigate the binding properties of glycopolymer decorated goldNP with influenza virus, all binding studies were performed by incubating aliquots of the goldNP with formalin inactivated PR8 influenza virus suspension for overnight at 4°C. Briefly, 2 µL of goldNP decorated with ω-2,6-sialyllactose-polymer were dispersed in 98 µL of PBS and then added to 100 µl of virus suspension which was prepared via dilution of virus (512 hemagglutinating units/µl) in PBS (1 to 10). After overnight culture, the change in size was performed via DLS measurement at 25°C. Lactose-conjugated goldNP were used as control to investigate the specific virus-binding property with virus.

**Formalin inactivation of PR8 influenza virus**

PR8 influenza virus (A/Puerto Rico/8/1934) virus was grown on MDCK cells in serum-free medium in the presence of TPCK-treated trypsin. Seven days after inoculation, culture medium was collected and centrifuged twice for 10 min at 450 g to remove cellular debris. Virions were then pelleted by centrifugation (20,000 g , 4°C, 16 h). Virus was inactivated by dissolving the pellets in 0.05 % formaldehyde (prepared from formalin stock) in PBS followed by continuous shaking for 7 days at 4 °C. Formalin-containing buffer was exchanged for PBS >10,000-fold (volume / volume) by sequential ultrafiltration with Vivaspin filtration columns (Sartorius Stedim...
Biotech, Aubagne Cedex, France) with 100 kDa cutoff. X47-WIV was stored at 4 ° C in the dark until used.
<table>
<thead>
<tr>
<th>Polymer composition</th>
<th>Mw [^{[1]}]</th>
<th>Mn [^{[1]}]</th>
<th>Dispersity[^{[1]}]</th>
<th>DP of HPMA[^{[2]}]</th>
<th>DP of APMA[^{[2]}]</th>
<th>Proportion of APMA(%) [^{[2]}]</th>
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\[^{[1]}\] data collected by HFIP SEC; \[^{[2]}\] data calculated based on \[^{1}H\]-NMR spectra;
Figure S1. (A) SEC traces of the different poly(HPMA-co-APMA) copolymers and their α-2,6-sialyllactose (abbreviated as SiLac) derivatives. (B) SEC traces of poly(HPMA$_{75\%}$-APMA$_{25\%}$)$_{DP100}$ and its lactose and α-2,6-sialyl lactose derivatives.
**Figure S2.** $^1$H-NMR analysis of the different poly(HPMA-co-APMA) copolymers and their lactose, respectively $\alpha$-2,6-sialyllactose derivatives. Note that only for the DP 100 25% APMA copolymer the lactose derivative was synthesized whereas for all polymers the $\alpha$-2,6-sialyllactose derivative was synthesized.

**Figure S3.** UV-VIS spectra of poly(HPMA-co-APMA) of sialyllactose modified poly(HPMA-co-APMA) after addition on the ninhydrin reagent, showing nearly full conversion of the primary amines.
Figure S4. TGA analysis of goldNP decorated with α-2,6-sialyllactose-polymer and sodium citrate.
Figure S5. QCM traces of the respective α-2,6-sialyllactose containing polymer followed by SNA or MAA adsorption on gold coated quartz chips. (B) quantification of the frequency shift upon adsorption of the α-2,6-sialyllactose containing polymer and SNA or MAA. n=3.