This version of the ESI published 03/11/2023 replaces the previous version published 14/12/2018. The equation on page 9 is replaced with a corrected version.

SUPPORTING INFORMATION

A Non-Sacrificial Method for the Quantification of Poly(ethylene glycol) Grafting Density on Gold Nanoparticles for Applications in Nanomedicine

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Experimental section

Materials

Hexadecyltrimethylammonium bromide (CTAB, ≥99%), gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.99%), sodium citrate tribasic dehydrate (citrate, ≥99%), sodium borohydride ($NaBH_4$, ≥98%), L-ascorbic acid (AA, ≥99.0%), silver nitrate (≥$AgNO_3$, 99.0%), and (3-aminopropyl)trimethoxysilane (APTMS, 97%) were purchased from Sigma-Aldrich. Sodium 2,2-dimethyl-isotope-2-silapentane-5-sulfonate (DSS) was purchased from Alading Reagent Inc. Deuterium oxide (D_2O, 99.9%) was obtained from Qingdao Teng Long Technology Co., Ltd. (Qingdao, China). Thiol-terminated poly(ethylene glycol) (HS-PEG, Mw of 2.0, 5.0, 10.0, and 20.0 kg/mol) and α-Methoxy-ω-carboxyl poly(ethylene glycol) (PEG-COOH, Mw of 5.0 kg/mol) were purchased from JenKem Technology (Beijing, China). Tetraethylorthosilane (TEOS) was purchased from Beijing Shiji (Beijing, China). α-Methoxy-ω-N-hydroxysuccinimide-polyethylene glycol (PEG-NHS, Mw of 5.0 kg/mol) and 2-[Methoxy(polyethyleneoxy)propyl] trimethoxysilane (PEG-silane, Mw of 5.0 kg/mol) were purchased from Peng Sheng Biological (Shanghai, China). All chemicals were used as received. Deionized water (18.2 MΩ) was used in all the experiments.

Synthesis of Gold Nanoparticles and Nanorods

The CTAB capped GNPs with average diameter less than 13 nm (8−13 nm) were synthesized based on the seed-mediated growth method reported by Murphy and co-workers.[1] The GNPs with sizes larger than 13 nm (13−43 nm) were obtained *via* the regrowth process to 13 nm GNPs by controlling the amount of $HAuCl_4$

and ascorbic acid in the regrowth solution. The citrate capped GNPs with a size of 13 nm were synthesized according to a reported method by Plech and co-worker.[2] The GNRs with an average length of 33.3 ± 2.95 nm and diameter of 7.05 ± 1.03 nm were prepared using the procedure reported by El-Sayed and co-workers.[3]

Attachment of HS-PEG on GNPs

The attachment of HS-PEG onto the surface of GNPs was achieved through the ligand exchange of HS-PEG with original capping ligand, *i.e.* citrate, CTAB, and PVP. For example, the GNPs (13 nm, capped by citrate) were concentrated by two cycles of centrifugation at 12000 rpm for 20 mins and redispersed into 250 μL D_2O solution, and then mixed with 250 μL HS-PEG (0.50 mg/mL, dissolved in D_2O). The mixture was immediately transferred into a clear NMR tube and characterized by NMR spectrometer to monitor the grafting process at 25 °C. The attachment of HS-PEG (in variable concentrations) with other size or capping ligand GNPs were performed in similar procedures.

Quantification of grafting density by Multi-Lorentzian-Splitting Algorithm

For quantification the σ of HS-PEG on the surface of GNPs *in situ*, we performed the algorithm of multi-Lorentzian-splitting to segregate the NMR peak of free PEG from the measured NMR spectra. Firstly, the measured ^{1}H NMR spectra need to be transformed into an ASCII text file by the software of MestReNova. Then, the NMR data with chemical shift in the range of 3.4−4.0 ppm were

collected, and the baseline was subtracted. The acquired data were imported into the code written by Python to calculate the number of components and corresponding NMR peak distribution following a standard procedure introduced on the website at https://github.com/Shirui816/MultipleDistributionFitting. The unknown ligand concentrations of free PEG were determined by comparing the integral of the NMR peak at 3.70 ppm with a standard curve of pure PEG in the concentration range of 0.05−5.00 mg/mL. From the view of information theory, models with the minimum BIC/AIC have the highest probability to give the actual observations, that is *g* of 3 in the case for 0.5 mg/ml PEG grafted to 13 nm citrated-caped NPs. However, choosing *g* of 2 did not yield a significant difference of the grafting densities but resulted in a grafting density of 1.44 chains/nm^2, which was slightly lower than the grafting density of 1.55 chains/nm^2 for *g* of 3. The grafting density of 1.55 should be more accurate and represents the actual grafting density, which was further verified through a post-centrifugation experiment that yielded a grafting density of 1.53 chains/nm^2.

SiO_2 Encapsulation of GNRs

The encapsulation of SiO_2 on GNRs was performed *via* TEOS hydrolysis.[4] Briefly, 6.0 μL of 20 wt.% TEOS in isopropanol was added in a 1.0 mL GNR solution ([GNR] ≈ 1 nM). The pH of the solution was adjusted to ~10.5 with 0.10 M of NaOH, and the solution was kept at 25 °C for 2 h under continuous stirring. Subsequently, another 2.0 μL of TEOS was added into the solution, and the mixture was stirred overnight. The mean length and diameter of the GNR@SiO_2

were 57.5 ± 5.23 and 27.4 ± 2.83 nm, respectively. The synthesized GNR@SiO_2 was purified by two centrifugation cycles at 8500 rpm for 15 mins with DI water.

The GNR@SiO_2-NH_2 was prepared *via* APTMS hydrolysis in the freshly prepared GNR@SiO_2 solution. 50 μL of APTMS was added into the as-synthesized 1.0 mL GNR@SiO_2 solution. The pH of the solution was adjusted to ~10 with 0.10 M of NaOH, and the solution was stirred at 25 °C overnight. The mean length and diameter of the GNR@SiO_2-NH_2 were 69.3 ± 5.94 and 44.1 ± 3.47 nm, respectively. The resultant GNR@SiO_2-NH_2 was purified by three cycles of centrifugation at 8500 rpm for 15 mins with DI water.

Attachment of PEG Chains to the Surface of GNR@SiO_2

The attachment of PEG chains to the surface of GNR@SiO_2 was performed through three typical routes. Specifically, route I utilized GNR@SiO_2-NH_2 to react with PEG-COOH in D_2O solution. 20 mL of GNR@SiO_2-NH_2 solution was concentrated by two cycles of centrifugation at 8500 rpm for 15 mins and redispersed into 250 μL D_2O, then mixed with 250 μL PEG-COOH (0.4 mg/mL, dissolved in D_2O). The mixture was reacted at 25 °C more than 24 h. Route II utilized PEG-NHS to react with GNR@SiO_2-NH_2 according to a reported procedure.[5] 4 mL GNR@SiO_2-NH_2 solution was concentrated and redispersed in 250 μL phosphate buffered solution (PBS) (5 mM PBS, pH = 7.3, phosphate dissolved in D_2O), then mixed with 250 μL PEG-NHS (0.20 mg/mL, dissolved in PBS). The reaction mixture was stirred at 25 °C overnight. Route III was performed *via* PEG-silane hydrolysis on the surface of GNR@SiO_2. 20 mL

GNR@SiO_2 solution was concentrated by two cycles of centrifugation at 8500 rpm for 15 mins and redispersed into 246 μL D_2O, then mixed with 250 μL PEG-silane (4.0 mg/mL, dissolved in D_2O). 4 μL HCl (1.0 mol/L) was added into the reaction solution. The mixture was then kept at 25 °C overnight.

Characterization

^{1}H NMR spectra were recorded by Bruker Avance 500 and 600 MHz instruments with sufficient relaxation delay of 3 seconds to allow complete relaxation between pulses at 298 K. All chemical shifts are relative to an external standard of sodium 2,2-dimethyl-isotope-2-silapentane-5-sulfonate (DSS) in a sealed capillary inside the NMR tube. Extinction spectra were measured with a PerkinElmer Lambda 950 UV−vis−NIR spectrometer with a data interval of 1 nm using 1 cm quartz cell. TEM imaging was performed with a JEOL JEM-2100F transmission electron microscope and a Hitachi H-800 transmission electron microscope operating at 200 kV and 175 kV, respectively, on carbon-coated TEM grids. The dynamic light scattering (DLS) experiment was performed by ALV/CGS-3 Compact Goniometer System using He-Ne laser operating at 632.8 nm. The DLS data was acquired by a 150 degree scattering angle at 25 °C. TGA was performed using TA Instruments Q-600 with a heating rate of 10 °C/min in an inert atmosphere (N_2) from 30 to 900 °C.

Discussion 1. Calculation of the Grafting Density by TGA

The average radius (R_{gold}) of the citrate capped GNPs was 6.37 ± 0.41 nm. The molecular weight of HS-PEG (MW_{PEG}) was 5.0 kg/mol. The density of bulk gold (ρ_{core}) is 19.3 g/cm^3. N_A is the Avogadro number. The weight fraction of PEG (W_{PEG}) and GNP

core (W_{gold}) were 22.6% and 77.4%, respectively, determined by the TGA measurement.

The grafting density (σ) can be calculated with the following equation:

$$\sigma = \frac{\frac{4}{3}\pi {R_{gold}}^3 W_{PEG} \rho_{core} N_A}{4\pi {R_{gold}}^2 W_{gold} MW_{PEG}} = \frac{R_{gold} W_{PEG} \rho_{core} N_A}{3\pi W_{gold} MW_{PEG}}$$

$$= \frac{6.37\ \text{nm} \times 22.6\% \times 19.3\ \text{g/cm}^3 \times 6.02 \times 10^{23} \text{chains/mol}}{3 \times 77.4\% \times 5000\ \text{g/mol}} = 1.44\ \text{chains/nm}^2$$

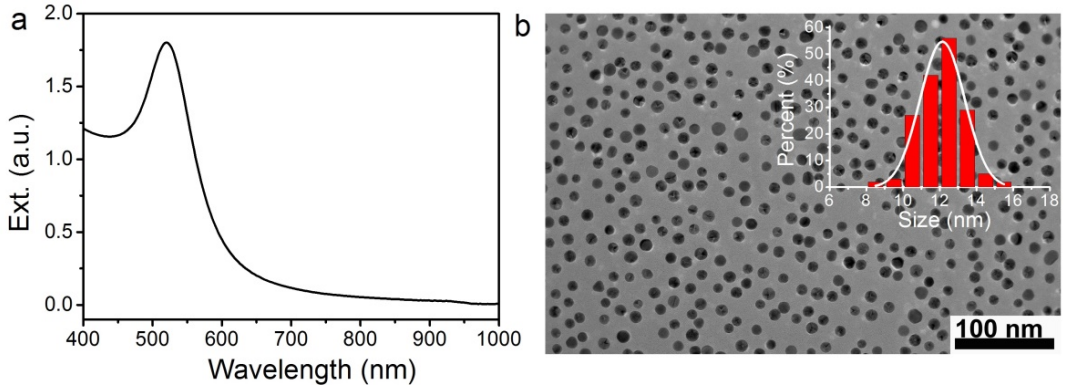

Fig. S1 Extinction spectrum (a) and TEM image (b) of the citrate capped GNPs for the grafting of HS-PEG.

Discussion 2. Calculation of the Molar Concentration of GNPs

The average diameter of GNPs was 12.74 ± 0.81 nm. The molar extinction coefficient (ε_{NP}) of the GNPs capped by citrate is 2.31×10^8 M^{-1} cm^{-1} calculated from a reported literature by Huo *et al.*[6] The light path length of the quartz cell was 0.10 cm. Therefore, the concentration of GNPs was calculated with Beer–Lambert law:

$$[\text{GNP}] = \frac{A}{\varepsilon_{GNP} \times b} = \frac{1.80}{2.31 \times 10^8\ \text{mol}^{-1}\text{cm}^{-1} \times 0.1\ \text{cm}} = 77.9\ \text{nM}$$

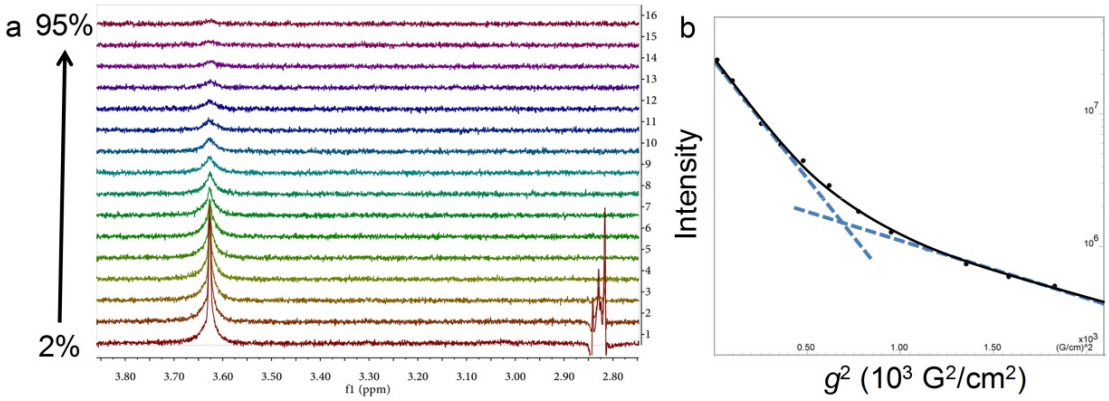

Fig. S2 (a) The ^{1}H NMR spectra of the diffusion-filtered spectra obtained with gradient amplitude varied from minimum (2%) to maximum (95%). (b) Decay of the intensity of the NMR signal at 3.70 ppm as a function of the square of the applied field gradient.

Discussion 3. Evaluation of PEG Components in Solution by Diffusion-Filtered Spectra

Fig. S2a and S2b show the attenuation of the resonance intensity at 3.70 ppm under the applied field gradient. The signal intensity decreased rapidly at low field gradient, and then the variations gradually became smaller with the increasing of field gradient. Fitting the plots of peak intensity with the square of applied field gradient, two linear lines with different slopes were observed, indicating the peak at the chemical shift of 3.70 ppm was consisted of two components of HS-PEG.

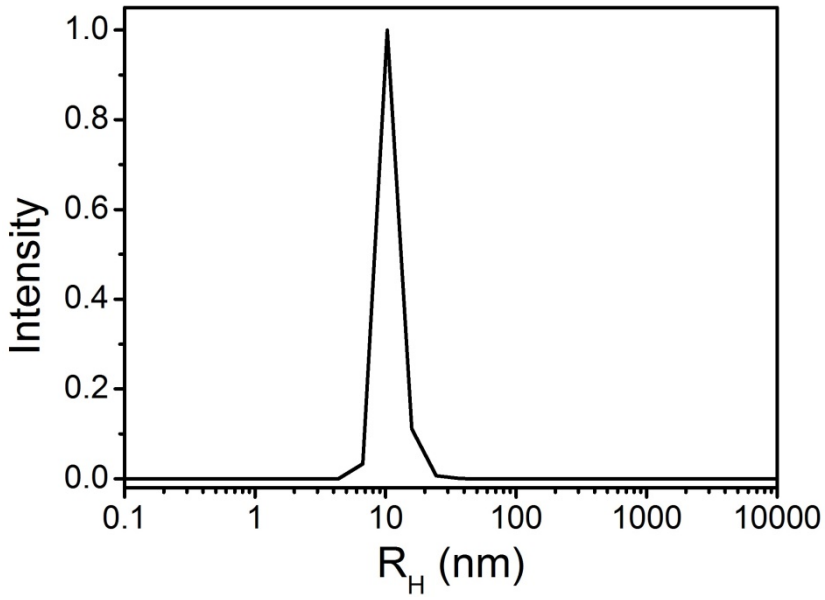

Fig. S3 Dynamic light scattering (DLS) spectrum of the 13 nm GNPs used for the grafting of HS-PEG.

Discussion 4. Calculation of the Diffusion Coefficient of GNPs by DLS

The average hydrodynamic diameter (d_H) of the GNPs was equal to 10.3 nm according to the DLS spectrum. The experiment temperature (T) was 298 K. The dynamic viscosity (η) of the D_2O was 1.1123 mPa·s. Hence, the diffusion coefficient (D) can be calculated by the Stokes–Einstein equation:

$$D = \frac{k_B T}{3\pi\eta d_H}$$

$$= \frac{1.38 \times 10^{-23}\,\mathrm{J/K} \times 298\,\mathrm{K}}{3 \times 3.14 \times 1.1123 \times 10^{-3}\mathrm{Pa \cdot s} \times 10.3 \times 10^{-9}\,\mathrm{m}} = 3.81 \times 10^{-11}\mathrm{m^2\,s^{-1}}$$

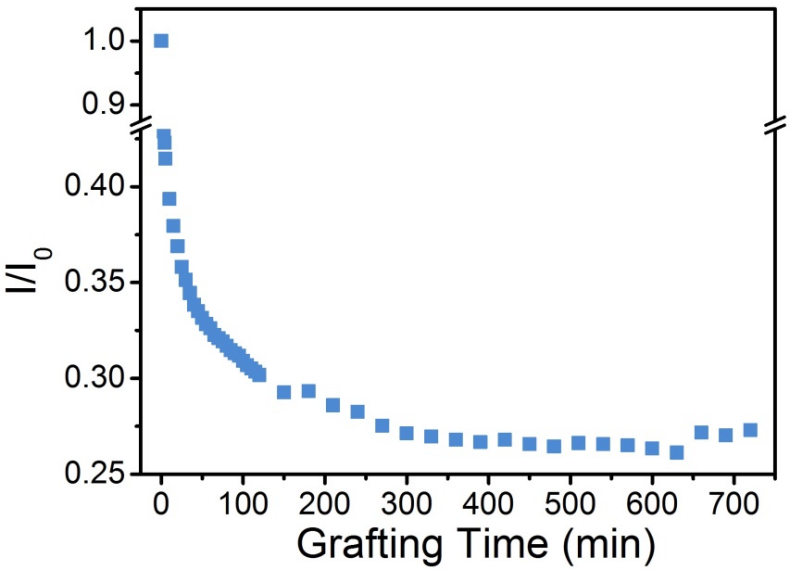

Fig. S4 Temporal evolution of integral areas of the peak at 3.70 ppm in the ^{1}H NMR spectra *versus* the grafting time of HS-PEG onto the surface of GNPs.

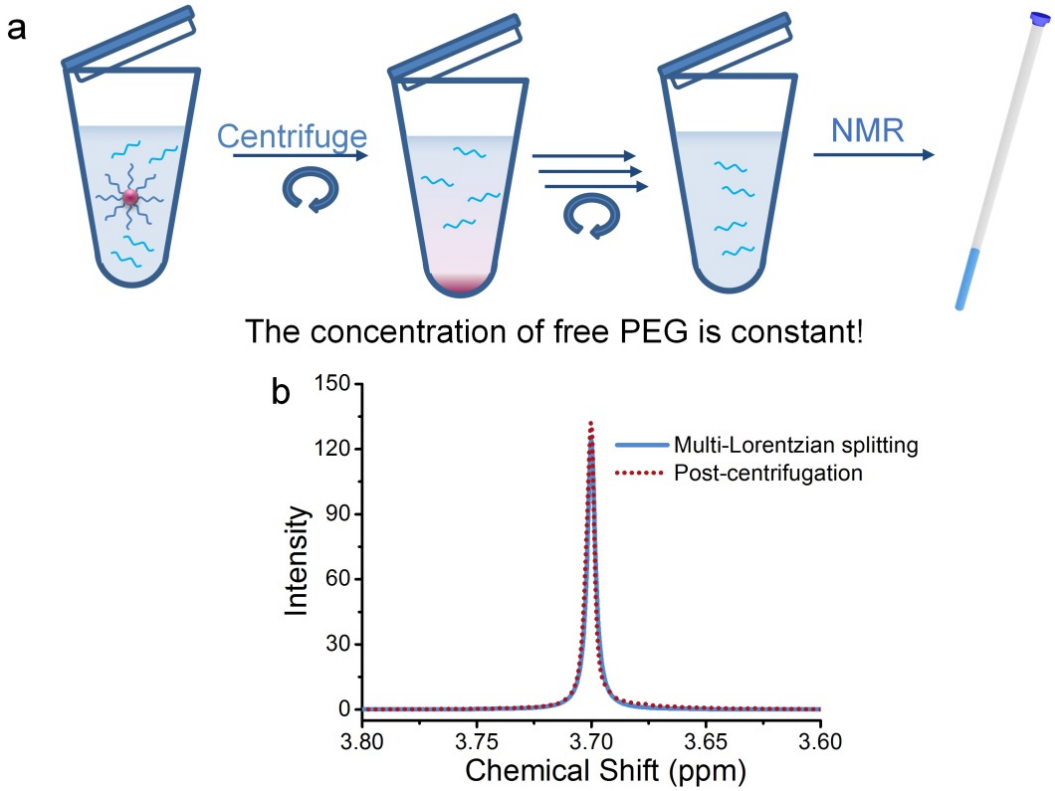

Fig. S5 (a) Scheme of the post-centrifugation strategy to isolate free PEG from grafted ones. In order to obtain the free PEG supernatant, multiple times of centrifugations were

used to completely remove the GNPs with grafted PEG from the solution. The grafting density was then quantified based on the ^{1}H NMR spectra of the free PEG supernatant. (b) ^{1}H NMR spectra of free PEG from the multi-Lorentzian- splitting algorithm and post-centrifugation experiments.

Table S1. Comparison of Free PEG Calculated from the Multi-Lorentzian-Splitting Algorithm and Post-Centrifugation Experiments

PEG type	υ_{HWHM} (ppm)	Integral area	Grafted mass (mg)	σ (chains/nm^2)
PEG_{pure}	0.00202	1.98734		
PEG_{free} *via* multi-Lorentzian-splitting	0.00210	0.81711	0.30787	1.55
PEG_{free} *via* post-centrifugation	0.00202	0.83220	0.30390	1.53

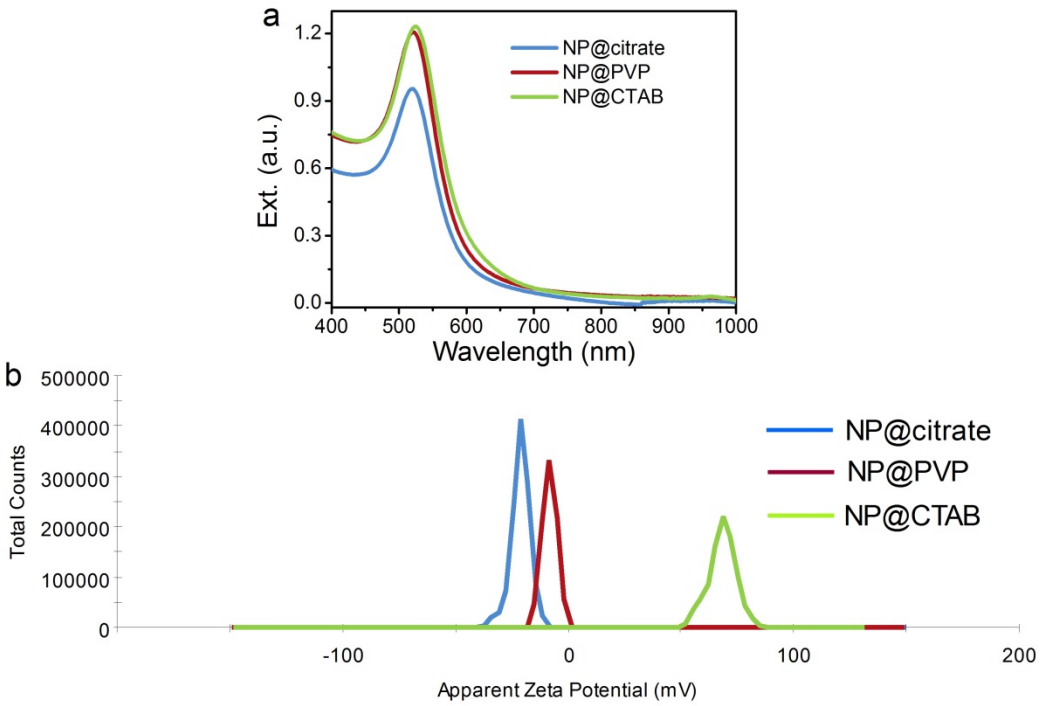

Fig. S6 Extinction (a) and zeta-potential (b) spectra of the GNPs capped by citrate, PVP, and CTAB.

Discussion 5. Characterization for NPs Capped by Citrate, PVP, and CTAB

The PVP and CTAB capped GNPs were prepared *via* the ligand exchange of CTAB and PVP with the citrate covered GNPs since CTAB and PVP possess stronger binding affinity with GNPs than that of citrate. The success of the ligand exchange of CTAB and PVP was thoroughly determined by extinction and zeta-potential measurements.

The peak positions of the surface plasmon resonance of PVP capped GNPs and CTAB capped GNPs red-shifted from 520 to 522 and 525 nm, and their peak intensities were increased by 26% and 29%, respectively, upon the replacement of citrate by PVP and CTAB on the surface of NPs (Fig. S5a). The zeta-potential of the GNPs changed from –21.1 to –8.1 and to 68.4 mV by replacing citrate with PVP and CTAB, respectively (Fig. S5b).

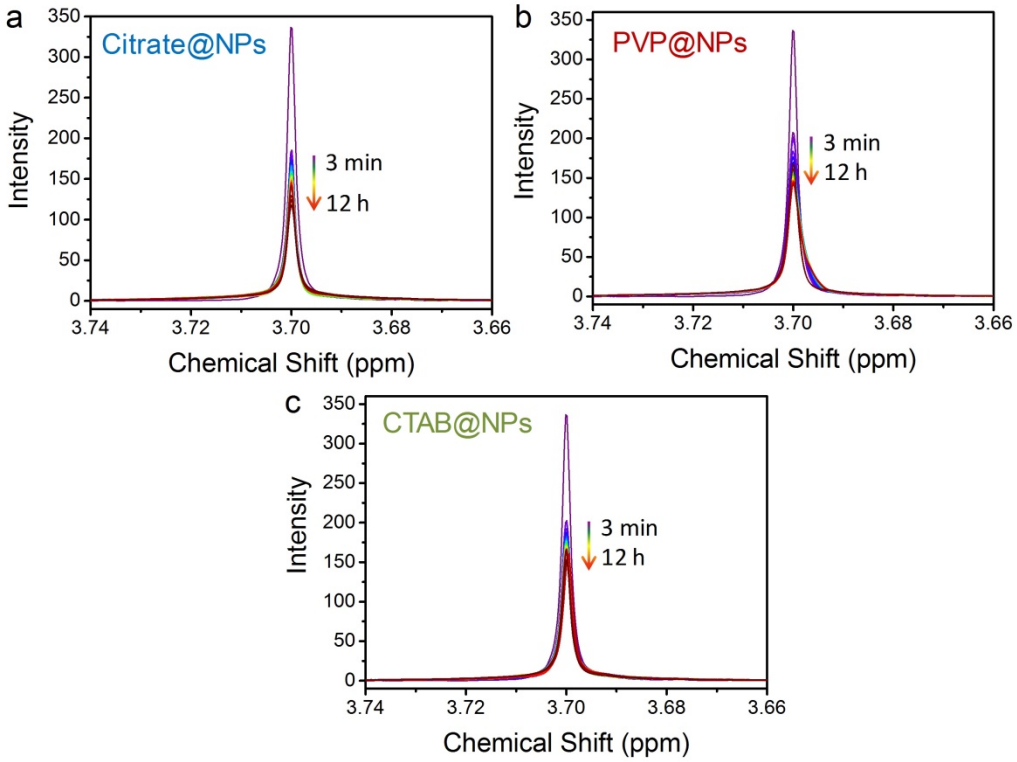

Fig. S7 Temporal evolution of ^{1}H NMR spectra of HS-PEG grafted GNPs capped by citrate (a), PVP (b), and CTAB (c), respectively.

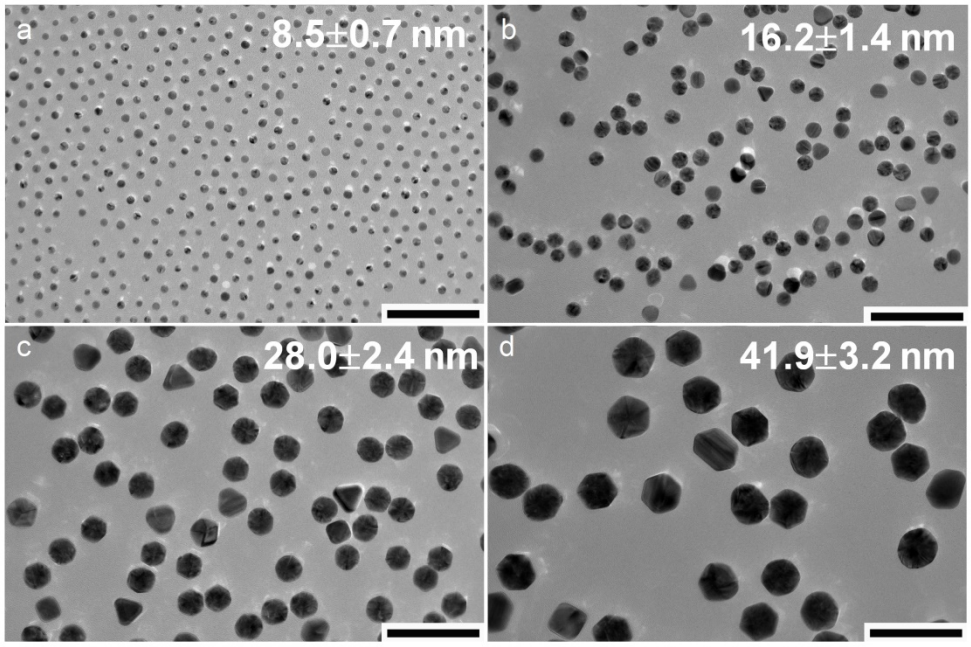

Fig. S8 TEM images of GNPs with average diameters of 8 (a), 16 (b), 28 (c) and 42 (d) nm, respectively. The scale bars: 100 nm.

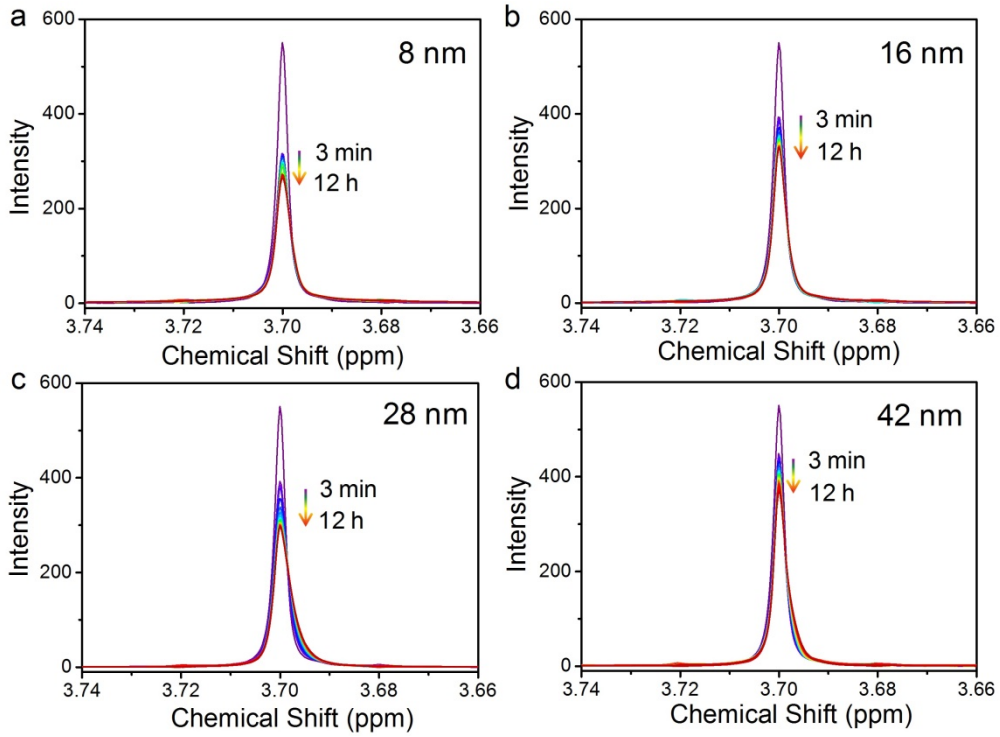

Fig. S9 Temporal evolution of ^{1}H NMR spectra of HS-PEG grafted onto GNPs capped by CTAB with average diameters of 8 (a), 16 (b), 28 (c) and 42 (d) nm, respectively.

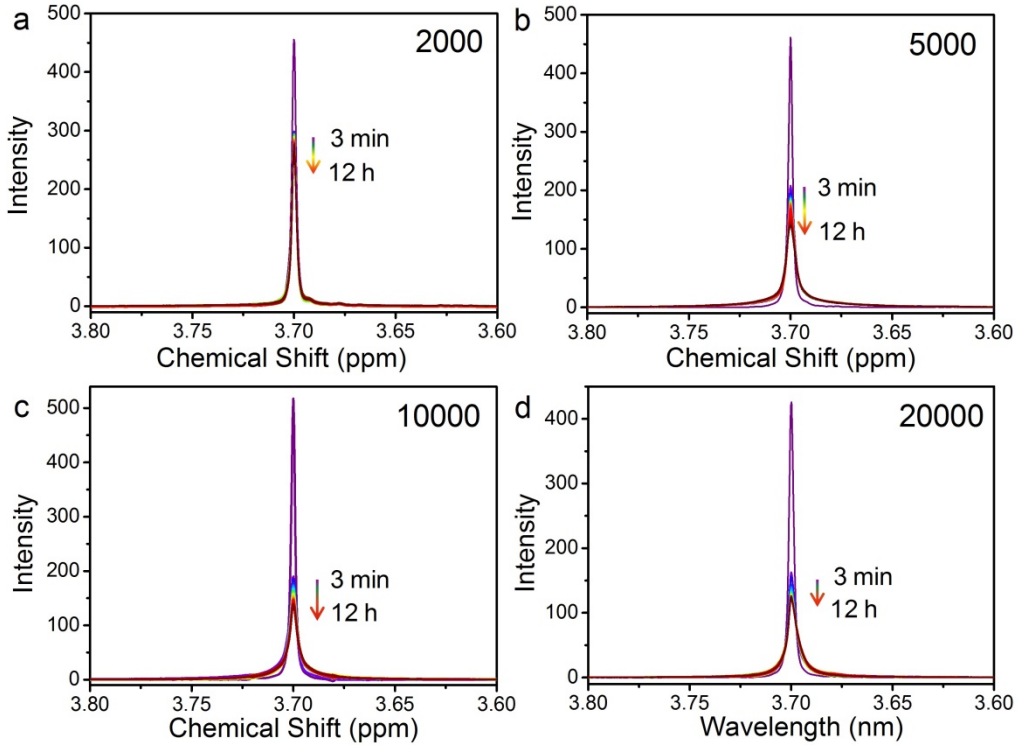

Fig. S10 Temporal evolution of ^{1}H NMR spectra of HS-PEG with variable molecular weights grafted onto GNPs (13 nm, capped by citrate). The molecular weight of HS-PEG was 2.0 (a), 5.0 (b), 10 (c), and 20 (d) kg/mol, respectively.

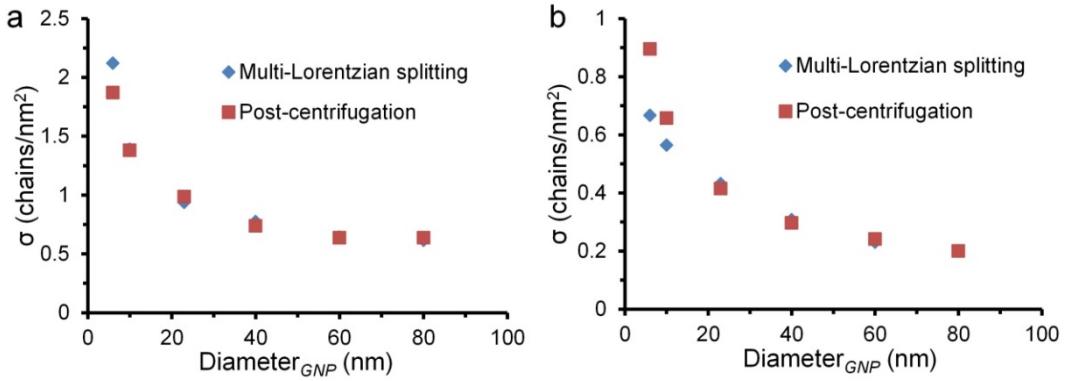

Fig. S11 Plots of grafting densities determined by the multi-Lorentzian-splitting algorithm and post-centrifugation methods for HS-PEG with molecular weight of (a) 5.0 (a) and 20 (b) kg/mol grafted to CTAB-capped NPs in a size range of 6–80 nm.

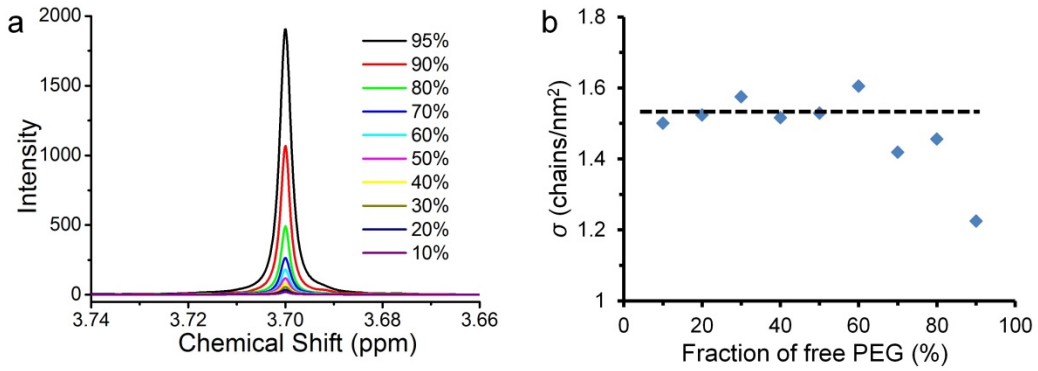

Fig. S12 (a) ^{1}H NMR spectra for various fractions of free PEG among all PEG added into the solution. (b) Plots of grafting densities determined by multi-Lorentzian-splitting algorithm with the fraction of free PEG. The dotted line in part b represents the grafting density determined by the post-centrifugation method (1.53 chains/nm^2).

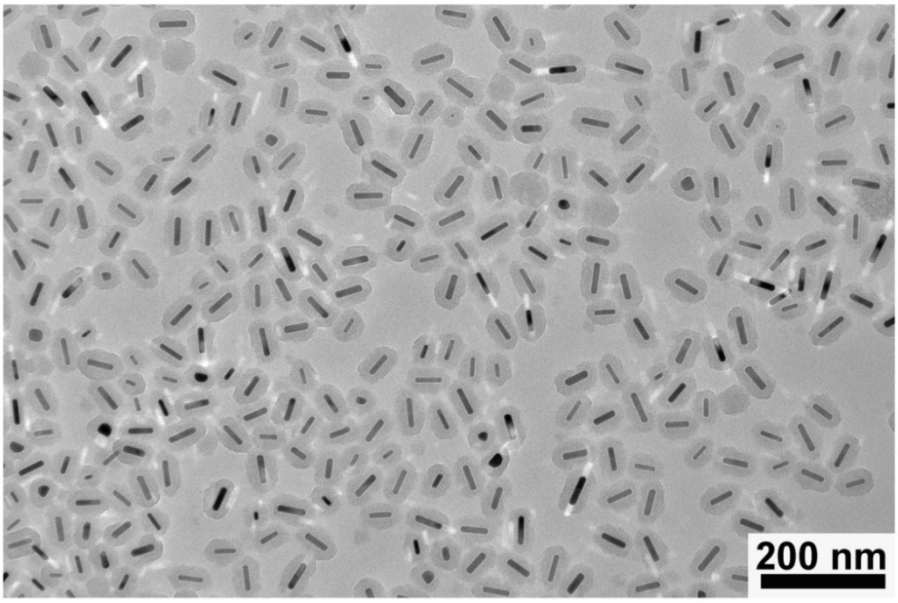

Fig. S13 TEM image of the GNR@SiO_2-NH_2 synthesized for the grafting of PEG-NHS. The mean length and diameter of the GNR@SiO_2-NH_2 were 69.3 ± 5.94 and 44.1 ± 3.47 nm, respectively.

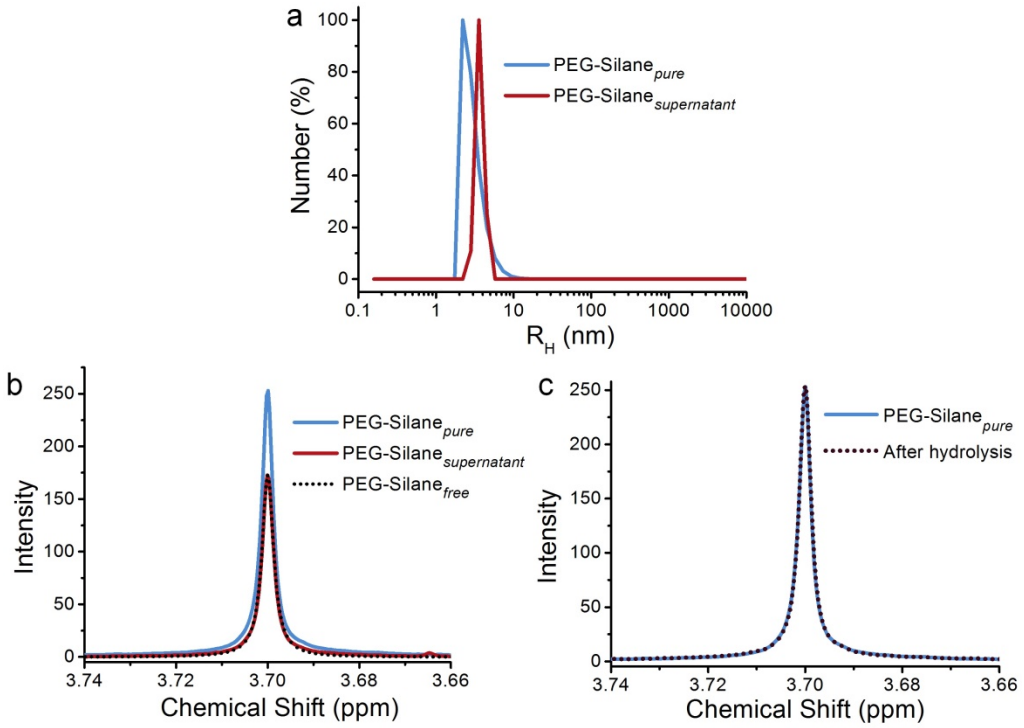

Fig. S14 (a) Dynamic light scattering (DLS) spectra of pure PEG and the supernatant of PEG-silane's hydrolysis solution (removing GNR@SiO_2 *via* the post-centrifugation strategy). (b, c) ^{1}H NMR spectra of PEG-silane hydrolyzed with (b) and without (c) GNR@SiO_2. The PEG-silane in the supernatant (PEG-silane$_{supernatant}$) and the PEG-

silane on the surface of GNR@SiO_2 (PEG-silane$_{grafted}$) were isolated *via* the post-centrifugation method. The free PEG-silane in solution (PEG-silane$_{free}$) was isolated *via* the multi-Lorentzian-splitting algorithm.

Table S2. Parameters of PEG NMR Peaks for Four Types of PEG-silane

PEG-silane type	υ_{HWHM} (ppm)	Integral area
PEG-silane$_{pure}$	0.00149	1.17027
PEG-silane$_{free}$ (*via* multi-Lorentzian-splitting)	0.00152	0.83058
PEG-silane$_{grafted}$ (*via* post-centrifugation)	0.01128	0.02977
PEG-silane$_{supernatant}$ (*via* post-centrifugation)	0.00150	0.82125

Discussion 6. The Absent of By-Product for the hydrolysis Reaction of PEG-silane

We do not observe the formation of PEG micelles during the hydrolysis reaction of PEG-silane under the reaction condition. The absence of PEG micelles was determined by both of the ^{1}H NMR and dynamic light scattering (DLS) for the supernatant of the reaction solution after the SiO_2-encapsulated GNRs were removed by the centrifugation at 8000 rpm for 15 min (It should be noted that this centrifugation rate is not high enough to remove PEG micelles from the reaction solution). The NMR peak of the supernatant (Fig. S14 and table S2) shows a half-width-at-half-maximum (υ) of 0.00150 almost as same with that of 0.00149 for pure PEG-silane molecules, indicating the protons on PEG molecules in the supernatant were in a similar dimension with that of pure PEG molecules. This was further approved by the DLS study, from which remained PEG molecules in the supernatant has a hydrodynamic radius of 3.67 nm similar with that of 2.89 nm for free PEG-silane. Additionally, in a controlled experiment without adding silica coated gold nanorods, both υ and integral of the NMR peak kept the same before and after hydrolysis reaction of PEG-silane, indicating the hydrolysis reaction can only graft on the surface of silica. Therefore, the formation of by-product PEG micelles during the hydrolysis of PEG-silane on SiO_2 encapsulated

GNRs can be negligible and does not lead to overestimate or underestimate for the calculated grafting densities.

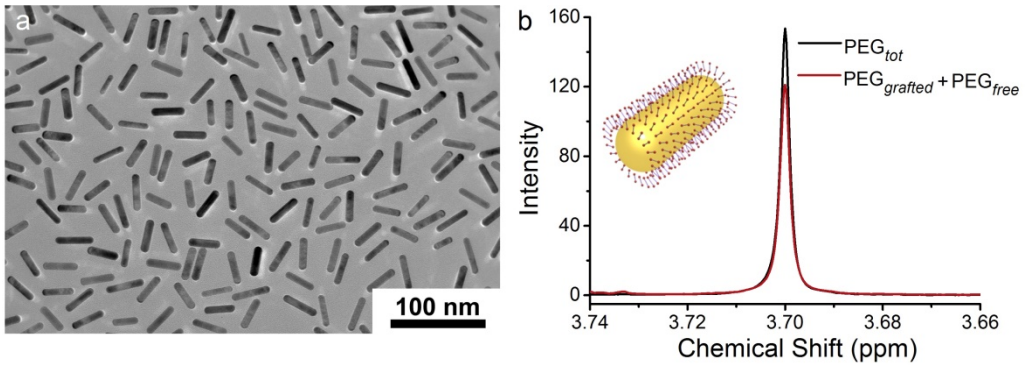

Fig. S15 (a) TEM image of the GNRs prepared for HS-PEG grafting. The average length and diameter of the GNRs were 33.3 ± 2.95 and 7.05 ± 1.03 nm, respectively. (b) ^{1}H NMR spectra of HS-PEG (5.0 kg/mol, 0.2 mg/mL) grafted onto the surface of GNRs (30.0 nM).

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