Supplementary Information Tumor Necrosis Factor Interaction with Gold Nanoparticles

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1. Experimental

1.1 Fluorescence Assay

Fluorescence spectrometry was conducted using a Quant-iT protein assay kit (Invitrogen, Paisley, UK).^{*} The assay involves a dye that fluoresces when bound to the detergent coating and/or hydrophobic regions of proteins, while the unbound dye is not fluorescent. First, 200 μ L of the working solution, containing the fluorescent dye and the protein buffer, was loaded into each microplate well, and then 5 μ L to 20 μ L test samples of the gold nanoparticle (AuNP)-tumor necrosis factor (TNF) conjugate were added into each well so that the fluorophore could bind to the protein. Bovine serum albumin (BSA) standards (10 μ L) with concentrations between 25 ng/ μ L and 500 ng/ μ L were placed in separate wells to facilitate quantitative calibration and were run at least in duplicate. A SAFIRE multi-detection monochrometer microplate reader (Tecan Inc, Durham, NC, U.S.A) was employed for the fluorescence measurements. The excitation wavelength was 470 nm and fluorescence was measured at 570 nm.

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After calibrating with the BSA standard, the amount of TNF in the AuNP samples was quantified based on the corresponding fluorescence intensity, $I_{\rm f}$. As shown in Figure S1a, the fluorescence was mostly determined by the concentration of proteins, regardless of the protein species (*i.e.*, TNF and BSA show a similar response for fluorescence versus concentration). Hence, we can use a BSA standard for the calibration. For this concentration range, the amount of TNF in each well was calculated using a correlation of $0.145I_{\rm f}^{0.854}$ (expressed as 10^{-9} g of TNF). Note that the correlation of fluorescence versus protein concentration may be varied, requiring calibrations for each 96-well plate measurement. In order to probe for AuNP assay interference, several controls were run with the test samples. The fluorescence intensity of TNF before and after adding AuNPs to the sample wells was measured. As shown in Figure S1b, the fluorescence intensity decreased as the AuNP concentration ($N_{\rm p}$) increased, , indicating that the assay may underestimate the amount of TNF when run with AuNPs. For improving accuracy, we have incorporated a fluorescence loss factor Y [=($I_{\rm f}$ with AuNP)/($I_{\rm f}$ when no AuNP)] into our quantitative analysis.



Figure S1. Fluorescence Assay. (a) Calibration lines of unbound TNF and BSA. R^2 >0.97. (b) Characterization of fluorescence loss factor, *Y*, versus the concentration of 60 nm-AuNP, N_p .

1.2 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy was performed using a Nicolet Spectra 750 FTIR spectrometer equipped with a Thunder Dome Germanium ATR accessory (Thermo Scientific, Madison, WI, U.S.A). In our experiments, we used liquid flow-cell mode (denoted as Type A) and dry-phase mode (denoted as Type B) ATR-FTIR. Schematic diagrams of the ATR-FTIR set-up are shown in Figure S2. Spectra were collected from 128 scans with a resolution of 1 cm⁻¹. For sample preparation, 800 μ L of AuNP solution was first concentrated to between 8 μ L and 20 μ L (*i.e.*, at least 40×) by centrifugation and then drop-cast onto the surface of the clean germanium ATR crystal. After evaporating the solvent in a clean bench at room temperature, a AuNP film was formed on the crystal surface.



Figure S2: Schematic of ATR-FTIR measurements.

For Type A measurements, a flow cell unit (Thermo Scientific) was attached to the top of the ATR crystal to allow fluid to contact and penetrate the AuNP film. Background spectra were recorded by introducing DI water into the flow cell. Solutions with various concentrations of molecular adsorbates were then introduced into the flow cell and the resulting spectra recorded. The experiments were performed at ≈ 21 °C using a sequential injection measurement: sample solutions containing selected ligands were injected sequentially into the flow cell ranging from low to high concentration. In Condition 1 (simultaneous competitive adsorption), ligands of interests (TNF and SH-PEG20K) were introduced to the AuNP film simultaneously. For the analysis of ligand displacement competitive adsorption (denoted as Condition 2), SH-PEG20K was introduced to displace the pre-existing TNF conjugates in the AuNP film. The concentrations of TNF and SH-PEG20K used in both conditions was 2.9 μ mol/L and 200 μ mol/L, respectively.

For Type B measurements, we first used centrifugation cleaning to remove the unbound ligands before drop-casting onto the Ge crystal to form the AuNP film. The cleaned AuNP films were then measured directly by ATR-FTIR under ambient conditions.

Because the IR beam path is controlled, providing a constant control volume V_T , the measured IR absorbance I_{ab} was calibrated against a known concentration of unbound molecules C_m . Assuming the IR absorption coefficients of molecules were unchanged upon adsorbing onto the AuNPs, the concentration of molecules present in V_T can be quantified. Based on the Beer-Lambert law, a linear correlation is expected between I_{ab} and C_m ,

$$I_{ab} = (k_{ab}l)C_m = k_{ab}^{*}C_m$$
 (S1),

where k_{ab} is molecular absorptivity, and l is the path length of the IR beam. To simplify the analysis without reducing accuracy, I_{ab} was determined by the measured height of the absorption peaks.

With I_{ab} , molecular surface density σ can be calculated using the following relationship that uses scaling of surface density for mercapto propionic acid (MPA),

$$\boldsymbol{\sigma} = \boldsymbol{\sigma}_{MPA,0} \frac{\boldsymbol{N}_{m}}{\boldsymbol{N}_{MPA}} = \boldsymbol{\sigma}_{MPA,0} \frac{\boldsymbol{I}_{ab} \boldsymbol{k}_{ab,MPA}}{\boldsymbol{I}_{ab,MPA} \boldsymbol{k}_{ab}^{*}} \quad (S2),$$

where $\sigma_{MPA,0}$ is the reference reported maximum surface density of MPA. $N_{\rm m}$ is the number of ligands in the control volume. $N_{\rm MPA}$, $I_{\rm ab,MPA}$ and $k_{\rm ab,MPA}^*$ are the $N_{\rm m}$, $I_{\rm ab}$, and $k_{\rm ab}^*$ of MPA at the same AuNP film thickness, respectively.¹ In our experiment, Eq. S2 may be simplified to

$$\boldsymbol{\sigma} = 0.235 \frac{\boldsymbol{I}_{ab}}{\boldsymbol{k}_{ab}^{*}} \quad (S3).$$

Using Eq. S3, we can characterize the surface density of 20 kDa thiolated polyethylene glycol (SH-PEG20K), TNF, and anti-TNF. For calculation of $\sigma_{\text{SH-PEG20K}}$, we measured I_{ab} for the (C-O)_{PEG} peak and then used $k_{ab}^* = 2.27$ in Eq. S3.¹ For characterizing the surface density of TNF and anti-TNF, we measured I_{ab} for the amide II band and used an approximation to obtain k_{ab}^* based on the previous results of BSA conjugates,^{1,2}

$$k_{ab,protein}^{*} = k_{ab,BSA}^{*} \frac{M_{m,protein}}{M_{m,BSA}}$$
(S4),

where $k_{ab,BSA}^* = 8.429$ and $M_{m,BSA} = 67$ kDa. From Eq. S4, $k_{ab,TNF}^* = 2.14$ and $k_{ab,anti-TNF}^* = 18.87$. Then, we used Eq. S3 to calculate the corresponding packing density.

1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Figure S3 demonstrates the procedure for our ELISA measurements, using the materials from the Human TNF- α ELISA kit (Thermo Scientific). First, AuNPs are reacted with TNF for at least 2 h. Then, we use centrifugation cleaning to remove the unbound TNF molecules. In the next step, biotinylated antibody reagents are added to the AuNP solution to interact with TNF conjugates on the surface. After 60 min, we perform another stage of centrifugation cleaning. Next, horseradish peroxidase (HRP)-conjugated streptavidin is added to react with the biotinylated anti-TNF on the AuNP surface. After 60 min, we perform an additional cycle of centrifugation cleaning. In the final step, the enzyme catalytic reaction is performed. We use tetramethylbenzidine (TMB) as the substrate catalyzed by the HRP attached to AuNPs. For a higher concentration of conjugated HRP, we expect to see a stronger catalytic effect reflected by the color change. After 30 min, we use the assay-supplied sulfuric acid to quench the reaction, and then separate the AuNPs from solution using centrifugation (80 krpm, 5 minutes). The supernatant containing the TMB substrates are measured by UV-Vis within 15 min after adding the sulfuric acid solution.



Figure S3: Schematic of ELISA procedures

2. Calculation of Gyration Radius

The radius of gyration for proteins, r_g , was calculated using the following equation derived by Narang *et al.*,³

$$r_{\rm g} = 0.395^* N^{3/5} + 7.257 \tag{S5},$$

where *N* is the number of amino acid groups per protein molecule; for the TNF monomer, N = 157. Hence, r_g is 1.6 nm for TNF monomer, 2.0 nm for TNF dimer, and 2.3 nm for TNF trimer. For the BSA monomer, N = 583, and thus r_g is 2.5 nm.

3. Characterization of Particle Size Distribution

ES-DMA is preferable for measuring particle size distribution of AuNPs, and the results have proved to be reasonably accurate in both mean size and distribution widths as demonstrated in the development of NIST SRM 8013.⁴ Figure S4a-b shows particle size distributions of AuNPs measured by ES-DMA, before and after molecular conjugation. The full width at half maximum, ≈ 10 nm, is unaffected by the conjugation of TNF or anti-TNF; this indicates that AuNPs are not destabilized or agglomerated by the procedure and that TNF conjugation occurs homogeneously without observable variations in packing density between different particles.

DLS results (Figure S4c-d) were also used for qualitative comparison. In general, the results of DLS agreed with and confirmed ES-DMA results following molecular conjugation with respect to: (1) increase of peak size; (2) size distribution shift without significant change in peak width. However, we choose to only report z-average particle size in the main text, based on the recommendation by ISO 22412:2008E [Particle size analysis - dynamic light scattering (DLS); 2008].



Figure S4: Particle size distributions of AuNPs, before and after molecular conjugation. (a) Particle size distributions of AuNPs measured by ES-DMA. Conditions were the same as used in Figure 3a of the main text. (b) Particle size distributions of AuNP measured by ES-DMA. Conditions were the same as used in Figure 4a of the main text. (c) Particle size distributions of AuNP measured by DLS. Conditions were the same as used in Figure 3a of the main text. (d) Particle size distributions of AuNP measured by DLS. Conditions were the same as used in Figure 4a of the main text. (d) Particle size distributions of AuNP measured by DLS. Conditions were the same as used in Figure 4a of the main text.

4. Spectra Analysis of Anti-TNF on AuNP and (TNF+AuNP)

As illustrated in Figure S5a, it would be necessary to induce TNF desorption to accommodate the adsorption of anti-TNF directly onto the limited gold surface, when σ_{TNF} is high. We estimated the possibility of this phenomenon through the spectra analysis of vibration frequency. As described in Figure S5b, vibration frequency of anti-TNF would have to be increased if the conjugated TNF was desorbed, showing contradicted with our experimental results (Figure 4b, main text, decrease of IR vibration frequency of anti-TNF). Hence it is

reasonable to exclude the effect of anti-TNF adsorption to citrate-stabilized gold surface directly when σ_{TNF} is high.



Figure S5: (a) Cartoon depiction of anti-TNF conjugation directly to AuNP to induce TNF desorption . (b) Estimation of vibration frequency of anti-TNF in Figure S5a.

References

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