Supporting Information

Time-Resolved Botulinum Neurotoxin A Activity Monitored using Peptide-Functionalized Au Nanoparticle Energy Transfer Sensors

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Experiments and Methods

Materials

The modified substrate peptide Cys-(PEG)$_{11}$-SNKTRIDEANQRATKXL-biotin was synthesized in Peptide Synthesis Core Facility of SBS, NTU. BoLcA (Product #610A), SNAPtide® (Product #521) unquenched calibration peptide for SNAPtide (Product #5281A2) were purchased from List Biological Labs, Inc. USA. PEG$_7$ thiol acid (Product #37156-0795) was purchased from Polypure AS, Norway. Streptavidin-Alexa 488 conjugate with labeling ratio of 3-6 dyes per protein was purchased from Invitrogen, Singapore.$^1$ 1.4 nm gold particles with single maleimide group were purchased from Nanoprobes, USA. Other chemicals were purchased from Sigma-
Aldrich without further notifications. Gold nanoparticles with an approximate average diameter of 6 nm were prepared by reduction of chloroauric acid with sodium borohydride. Typically, 0.5 ml of 0.1 M HAuCl$_4$ trihydrate in water and 2 ml of 0.025 M sodium citrate in water were added to 187.5 ml of pure water (MilliQ, 18.2 MΩ cm) and stirred. Next, 5 ml of 0.1 M NaBH$_4$ was added and the solution color changed from colorless to orange. Stirring was stopped and the solution was left undisturbed for 2 h before use. Gold nanoparticles with an approximate average diameter of 18 nm were prepared similarly. Briefly, 12 ml HAuCl$_4$ (2.54 mM) was added to 102 ml pure water under stirring and brought to boil in a round-bottom flask with a condenser to maintain a constant volume of the reaction mixture. Upon boiling, 6 mL of 10 mg/ml sodium citrate was added and continued boiling for another 30 minutes under stirring until the solution turned to red color.

**Peptide functionalization of AuNPs (AuNP-pep)**

The PEG$_7$ thiol acid (aPEG) served as the stabilizer molecules was mixed with the peptide substrate at the concentration ratios (peptide/aPEG of 1:100 and 0.5:100) in 50mM HEPES buffer (pH 7.4, 0.05% TWEEN20). Then a same volume of 18 nm AuNPs (~0.5 nM) was added into the mixture and incubated for 2 hours. The final concentration of the aPEG molecules was 25µM. To remove the unbound peptide and aPEG, the AuNPs were repeatedly centrifuged and resuspended in fresh 50mM HEPES buffer until estimated free peptide concentration in the solution became less than 1 pM. The purified AuNP-pep were then concentrated and stored at 4 °C until use. The 6 nm AuNPs were modified with peptide similarly but purified through PD minitrap G-25 column (GE healthcare).

The modification of 1.4 nm AuNPs with peptide was carried out according to the protocol provided by Nanoprobes. Briefly, Adding the freshed dissolved AuNPs (30 nmol in 1 mL water)
into the peptide to achieve the peptide concentration of 6 nmol/ml. The mixture solution was incubated for overnight at 4 °C. The unbound peptides were separated from the gold particles-peptide conjugates using PD minitrap G-25 column. The conjugate was eluted through the column with 0.01 M PBS buffer and collected as the colored AuNPs solution. The final concentration of the gold particles-peptide conjugates were estimated as 10 µM.

**Detection assay of BoLcA**

The AuNP-pep conjugates were incubated with BoLcA at various concentrations under 37 °C. After a specific period of incubation, streptavidin-Alexa conjugates were incubated with the mixture for 15 min prior to the measurement of the fluorescence intensity. The fluorescence intensity of Alexa 488 was measured with the fluorescence spectrometer (Fluorolog, Horiba, USA) at the excitation wavelength of 470 nm. Lifetime measurement of Alexa 488 was carried out with a LED pulse laser (wavelength of λ=405 nm, 100 ps) at the emission wavelength of λ\text{em}=520 nm.

**Enzyme activity characterization**

The catalytic reaction of enzymes E with the substrates S to form product P is typically follows the following model

\[
E + S \underset{k_{-1}}{\overset{k_i}{\rightleftharpoons}} ES \rightarrow E + P, \tag{1}
\]

where ES is the enzyme-substrate complex. With the steady-state assumption, the concentration of the intermediates [ES] stays a constant \((d[ES]/dt=0)\). The initial rate \(v\) is expressed as the Michaelis-Menten equation:\(^2\)

\[
v = \frac{d[P]}{dt} = \frac{k_{cat}[E]_0[S]}{(k_i + k_{cat}) / k_i + [S]} = \frac{k_{cat}[E]_0[S]}{K_M + [S]}, \tag{2}
\]
where $K_M$ is the Michaelis constant, $k_1$ and $k_{-1}$ are the rates for ES complex association and dissociation, $k_{\text{cat}}$ is the turnover number, $[S]$ and $[E]_0$ are the concentration of substrate and the total concentration of enzyme, respectively. Under the condition where $[S] \leq K_M$ and $[S] = [S]_0 - [P]$, equation 2 can be integrated to give the time-dependent substrate concentration, equation 3, in terms of $K_M$, $k_{\text{cat}}$ and time $t$,

$$P(t) = A + B \left(1 - e^{-k_{\text{cat}}[E]_0 t}\right),$$

where $A$ and $B$ are the constants, $K_T = k_{\text{cat}}/K_M$ is considered as the specificity constant to indentify the enzyme catalytic efficiency. This equation is predicated on the assumption that the enzyme is stable over the time course, the reaction is irreversible and the product is not an inhibitor.

**FRET calculation**

The energy transfer for large AuNPs with diameter of 6 nm and 18 nm were assumed to be dominated by FRET, from which the characteristic radius ($R_0$) is estimated as $R_0 = 16.6$ and 29.8 nm, respectively, according to the following equation,$^3$

$$R_0 = 0.211 \left[ k^2 n^{-3} \Phi_D J(\lambda) \right]^{1/6},$$

where $k^2$ is the orientation factor, $n$ is the refractive index of the medium, $\Phi_D$ is the quantum yield of the donor dye. The overlap integral $J(\lambda)$ between the AuNPs absorption spectrum and SA488 emission spectrum (Fig. 2) are $1.42 \times 10^{18}$ and $4.74 \times 10^{19}$ M$^{-1}$ cm$^{-1}$ nm$^4$ for 6 nm and 18 nm, respectively. $J(\lambda)$ is calculated from the normalized fluorescence intensity $F_D(\lambda)$ of the donor in the absence of acceptor and extinction coefficient of AuNPs $\varepsilon_{\text{AuNPs}}(\lambda)$ using the following equation,

$$J(\lambda) = \int F_D(\lambda) \varepsilon_{\text{AuNPs}}(\lambda) \lambda^4 d\lambda,$$
where maximum extinction coefficients of gold nanoparticles with diameter of 6 nm and 18 nm are $\varepsilon_{\text{Au6}}=1.89\times10^7$ and $\varepsilon_{\text{Au18}}=7.27\times10^8$ M$^{-1}$ cm$^{-1}$, respectively, as reported.\(^4\)

**Supporting Figures**

Figure S1. MALDI-TOF results: (a) 10 $\mu$M peptide. (b) 10 $\mu$M peptide after 20 nM BoLcA cleavage at 37°C for 3 hours. The 3013.3 peak indicates the full-length of peptide, while the 1054.6 and 1977.8 peaks indicate the C-terminal and N-terminal half respectively.
Fig. S2. The size distribution of 6 nm and 18 nm AuNPs.

Fig. S3. The fluorescence quenching efficiency for SA488 incubated with 6 nm AuNPs with various thiol-peptide/thiol-PEG ratios (1) 0.01, (2)0.005, (3)0.002.
Fig. S4. Absorbance spectra of 6 nm AuNP (1) before and (2) after mixing with SA488 at the AuNP-pep/SA488 molar ratio of 3:1. The decrease of absorbance intensity is ascribed to the dilution of AuNP after addition of SA488.

(1) http://tools.lifetechnologies.com/content/sfs/COAPDFs/2014/1531692_S11223.pdf.