Supporting Information – A near-infrared, surface-enhanced, fluorophore-linked immunosorbent assay

Michael D. Furtaw, David L. Steffens, Teresa M. Urlacher, Jon P. Anderson*

LI-COR Biosciences, Inc., Lincoln, Nebraska 68504

E-mail: mike.furtaw@licor.com

Table of Contents

1. PROTOCOL – DIRECT SEFLISA (RABBIT IGG)

A. Antigen Immobilization

a. Complete a two-fold serial dilution of rabbit IgG to obtain samples between 1000 pg/ml and 2 pg/ml. The buffer is carbonate-bicarbonate with a pH between 9.2 and 9.8.
b. Add 100 µl of each sample to the appropriate wells in the 96-well microtiter plate.
c. Cover plate and incubate overnight at 4°C.

B. Blocking

a. Invert plate and shake out antigen.
b. Wash each well in plate with 300 µl of PBST 2 times and PBS 1 time for 1 minute each on a plate shaker.
c. Add 300 µl of Odyssey blocking buffer to each well.
d. Cover plate and incubate for 1 hour at room temperature on a plate shaker.

C. Detection Antibody

a. Invert plate and shake out blocking buffer. No need to wash at this point.
b. Dilute biotinylated anti-rabbit conjugate in blocking buffer to a concentration of 1 µg/ml.
c. Add 100 µl of diluted detection antibody to each well.
d. Cover plate and incubate for 1 hour at room temperature on a plate shaker.

D. Streptavidin – Fluorophore Conjugate

a. Invert plate and shake out detection antibody.
b. Wash each well in plate with 300 µl of PBST 2 times and PBS 1 time for 1 minute each on a plate shaker.
c. Dilute SAv-800CW (1 mg/ml) in blocking buffer to a concentration of 0.1 µg/ml.
d. Add 100 µl of diluted SAv-800CW to each well.
e. Cover plate with seal and foil, incubate for 1 hour at room temperature on a plate shaker.

E. Protease K Solution

a. Invert plate and shake out streptavidin – fluorophore conjugate.
b. Wash each well in plate with 300 µl of PBST 1 time, PBS 1 time, and dH2O 1 time for 1 minute each on a plate shaker.
c. Dilute protease K salt solution (250X) by 1:250 in dH2O.

d. Add 50 µl of diluted protease K salt solution to each well. Note: 50 µl of dH2O was added to reference (FLISA) wells instead.
e. Cover plate with seal and foil, incubate for 30 minutes at room temperature on a plate shaker.

F. AgNP Solution

a. Add 50 µl of AgNP solution to each well. Note: 50 µl of dH2O was added to reference (FLISA) wells instead.
b. Cover plate with seal and foil, incubate for 15 minutes at room temperature on a plate shaker.
c. Detect on Odyssey Sa with a focus offset of 3.4 mm at an intensity of 11.

2. PROTOCOL – SANDWICH SEFLISA (AFP)

A. Capture Antibody

a. Dilute mouse anti-human AFP in PBS to a concentration of 2 µg/ml.
b. Add 100 µl of diluted capture antibody to each well in the 96-well microtiter plate.
c. Cover plate and incubate overnight in drawer (dark) at room temperature.

B. Blocking

a. Invert plate and shake out excess capture antibody.
b. Wash each well in plate with 300 µl of PBST 2 times and PBS 1 time for 1 minute each on a plate shaker.
c. Add 300 µl of Odyssey blocking buffer to each well.
d. Cover plate and incubate for 1 hour at 37°C on a plate shaker.

C. Antigen

a. Invert plate and shake out blocking buffer.
b. There is no need to wash at this point.
c. Complete a two-fold serial dilution of human AFP to obtain samples between 5000 pg/ml and 9.8 pg/ml. The diluent is 1% BSA in PBS.
d. Add 100 µl of each sample to the appropriate wells in the 96-well microtiter plate.
e. Cover plate and incubate for 1 hour at 37°C on a plate shaker.

D. Detection Antibody

a. Invert plate and shake out sample.
b. Wash each well in plate with 300 µl of PBST 2 times and PBS 1 time for 1 minute each on a plate shaker.
c. Dilute biotinylated chicken anti-human AFP in blocking buffer to a concentration of 0.2 µg/ml.
d. Add 100 µl of diluted detection antibody to each well.
e. Cover plate and incubate for 1 hour at 37°C on a plate shaker.

E. Streptavidin – Fluorophore Conjugate

a. Invert plate and shake out detection antibody.
b. Wash each well in plate with 300 µl of PBST 2 times and PBS 1 time for 1 minute each on a plate shaker.
c. Dilute SAv-800CW (1 mg/ml) in blocking buffer to a concentration of 0.1 µg/ml.
d. Add 100 µl of diluted SAv-800CW to each well.
e. Cover plate with seal and foil, incubate for 1 hour at 37°C on a plate shaker.

F. Protease K Solution

a. Invert plate and shake out streptavidin – fluorophore conjugate.
b. Wash each well in plate with 300 µl of PBST 1 time, PBS 1 time, and dH2O 1 time for 1 minute each on a plate shaker.
c. Dilute protease K salt solution (250X) by 1:250 in dH2O.
d. Add 50 µl of diluted protease K salt solution to each well. Note: 50 µl of dH2O was added to reference (FLISA) wells instead.
e. Cover plate with seal and foil, incubate for 30 minutes at 37°C on a plate shaker.

G. AgNP Solution

a. Add 50 µl of AgNP solution to each well. Note: 50 µl of dH2O was added to reference (FLISA) wells instead.
b. Cover plate with seal and foil, incubate for 15 minutes at 37°C on a plate shaker.
c. Detect on Odyssey Sa with a focus offset of 3.4 mm at an intensity of 11.

3. PROTEASE EFFICIENCY

A. Purpose

An experiment was performed to determine the efficiency of the protease K at remobilizing the SAv-800CW (i.e. un-tethering it from the surface of the microtiter plate).

B. Procedure

a. Conduct a two-fold dilution series of biotinylated bovine serum albumin (b-BSA) to create 10 samples between 20,000 and 39 ng/ml. The diluent is 40 µM BSA in 0.1X PBS.

b. Add 100 µl of each sample to four wells in the corresponding columns of a 96-well microtiter plate. Cover the plate and incubate for 1 hour at room temperature on a plate shaker.

c. Invert plate and dump out sample. Wash each well in the plate with 300 µl of PBS 3 times for 2 minutes each on a plate shaker. Add 300 µl of Odyssey blocking buffer to each well. Cover plate and incubate for 1 hour at room temperature on a plate shaker.

d. Invert plate and dump out blocking buffer. Add 100 µl of SAv-800CW diluted 1:10,000 in Odyssey blocking buffer to each well. Cover plate with seal and foil, incubate for 1 hour at room temperature on a plate shaker.

e. Invert plate and dump out SAv-800CW solution. Wash each well as before. Dilute protease K salt solution (250X) 1:250 in dH2O. Add 50 µl of protease K solution to each well in 2 rows of the plate. Add dH2O to the other 2 rows. Cover plate with seal and foil, incubate for 30 minutes at room temperature on a plate shaker.

f. Invert plate and dump out protease solution. Wash each well as before. Add 50 µl of dH2O to each well. Scan on Odyssey Sa with a focus offset of 3.4 mm at an intensity of 9.

C. Results

Figure S1 shows the cleavage efficiency of the protease K solution at the various concentrations of immobilized b-BSA. The asymptotic value is ~ 94%. As expected, the efficiency decreases at very low values of b-BSA where the concentration is insignificant in comparison to the blocking buffer proteins.

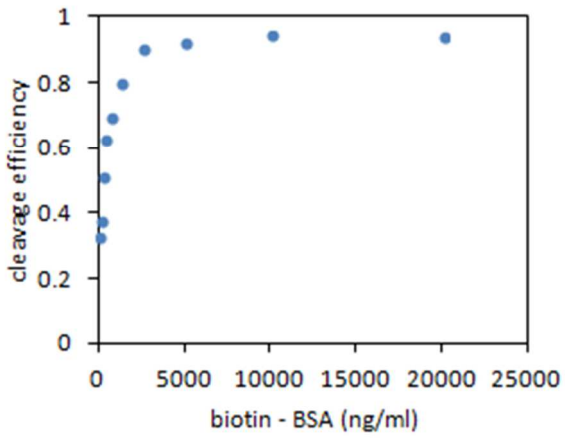

Figure S1. Cleavage efficiency of protease K solution at various concentrations of immobilized biotinlyated BSA. Cleavage efficiency is defined as the percent of SAv-800CW remobilized after protease K treatment, which was measured as the ratio of fluorescence from wells with protease K divided by the reference wells which contained only dH2O.

4. SDS-PAGE ANALYSIS OF ASSAY SUPERNATANT

A. Purpose

An experiment was performed to determine the form of the SAv-800CW after protease treatment. Specifically, to determine if the protease cleaves the SAv itself and whether or not the SAv becomes immobilized alone or as a complex with antibody attached.

B. Procedure

a. Follow the protocol for a direct SEFLISA (as shown in Section 1), except for the final section using AgNP, for a 10-fold dilution series of rabbit IgG from a concentration of 1000 to 0.001 ng/ml.

b. Immediately after the protease treatment, collect 30 µl of solution from each well. Add 10 µl of loading buffer to each sample. Incubate the samples at 70°C for 10 minutes in order to denature the proteins.

c. Perform SDS-PAGE with each sample using NuPAGE 4-12% Bis-Tris gels. Also load the following for comparison (with the same pre-treatment):

i. SAv-800CW (1:10000 dilute)

ii. 800CW ladder in two columns

d. Scan on Odyssey Classic.

C. Results

Comparing the sample lanes to the SAv-800CW alone (figure S2), it appears that the majority of SAv-800CW is immobilized without remaining attached to antibody. Some fluorescence appears smeared in the size range between 50 and 250 kDa, which is probable due to SAv-800CW remaining attached to various forms of antibody, fragments and whole. The majority of the signal shows up in similar bands as the SAv-800CW reference sample which indicates monomers, dimers, trimers, and tetramers of the SAv-800CW.

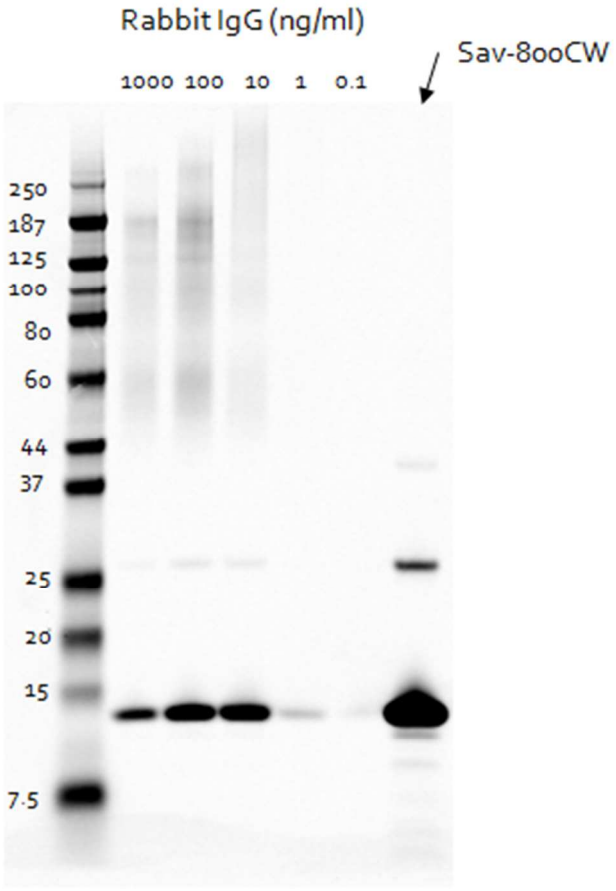

Figure S2. Fluorescence image of SDS-PAGE gel showing the distribution of SAv-800CW after protease treatment.

5. CALCULATION – QUALITY FACTOR

As mentioned in the text, the quality factor of a plasmonic system represents the number of plasmon oscillations, prior to decay, per excitation oscillation. A high quality factor represents local electromagnetic field enhancement with relatively low internal dissipative losses[1]. The quality factor (Q) can be calculated by

$$Q = \frac{\omega}{2\gamma} \approx \frac{\omega \frac{\delta \mathrm{Re}\varepsilon_m(\omega)}{\delta \omega}}{2\mathrm{Im}\varepsilon_m(\omega)}$$

where ω is the angular frequency (s^{-1}), γ is the decay rate (s^{-1}), and ε_m is the complex permittivity of the metal nanoparticle. Notice that the quality factor is only a function of the frequency and permittivity of the nanoparticle material[1] and therefore remains unchanged during the aggregation process (even though the far-field resonance broadens). Empirical data[2] were used for the permittivity of silver in our calculation.

6. SEM IMAGES OF AGGREGATES

The following images were acquired after forming aggregates using the AgNP solution mixed with 5 mM NaCl. These images are intended to provide verification of the aggregation process as well as visual representations of the aggregate size and shape. The aggregates in solution may differ as it is very difficult to immobilize aggregates without affecting the aggregation process. Notice that some of the AgNP monomers appear much larger than the 20 nm average. This may be due to agglomeration where the AgNP monomers combine to form larger spherical particles that appear as original monomers.

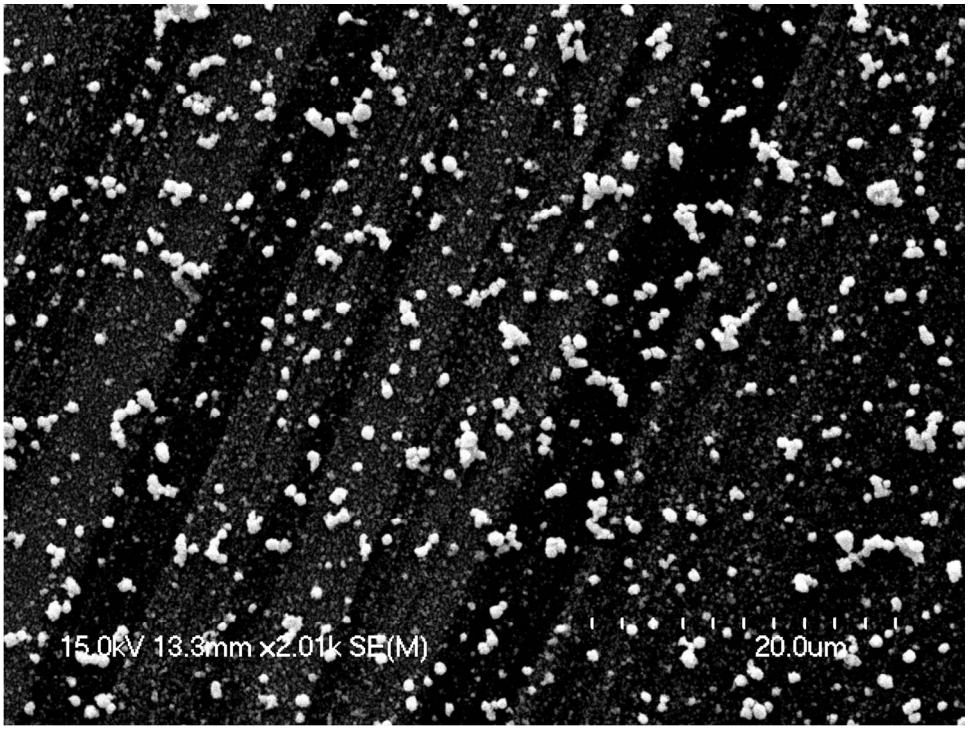

Figure S3. SEM image of AgNP aggregates formed with 5 mM NaCl.

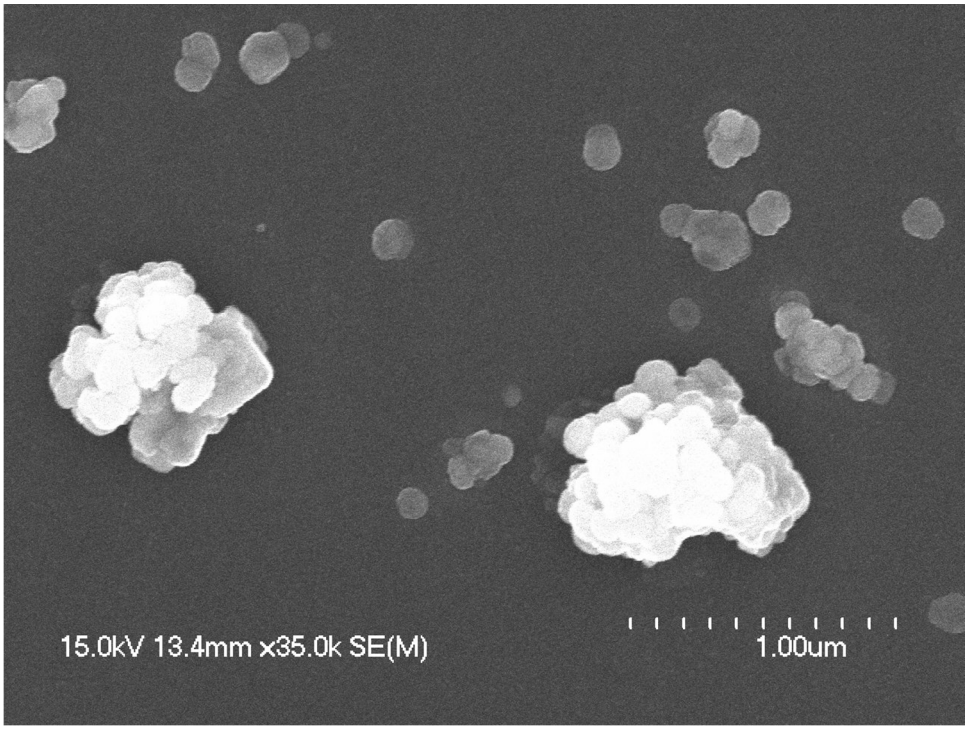

Figure S4. SEM image of AgNP aggregates formed with 5 mM NaCl at higher magnification.

7. REFERENCES

(1) Stockman, M. I. *Opt. Express* **2011,** 19, 22029-22106.
(2) Johnson, P. B.; Christy, R. W. *Phys. Rev. B* **1972,** 6, 4370-4379.