

Video Article

A Rapid and Quantitative Fluorimetric Method for Protein-Targeting Small Molecule Drug Screening

Yong Yu¹, Siu Yee New¹, Jiaxian Lin², Xiaodi Su¹, Yen Nee Tan¹

¹Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR)

²School of Chemical & Biomedical Engineering, Nanyang Technological University

Correspondence to: Yen Nee Tan at tanyn@imre.a-star.edu.sg

URL: <https://www.jove.com/video/53261>

DOI: [doi:10.3791/53261](https://doi.org/10.3791/53261)

Keywords: Bioengineering, Issue 104, Gold nanoclusters, synthesis, human serum albumin, fluorescence, drug screening, binding constant, stability, protein unfolding

Date Published: 10/16/2015

Citation: Yu, Y., New, S.Y., Lin, J., Su, X., Tan, Y.N. A Rapid and Quantitative Fluorimetric Method for Protein-Targeting Small Molecule Drug Screening. *J. Vis. Exp.* (104), e53261, doi:10.3791/53261 (2015).

Abstract

We demonstrate a new drug screening method for determining the binding affinity of small drug molecules to a target protein by forming fluorescent gold nanoclusters (Au NCs) within the drug-loaded protein, based on the differential fluorescence signal emitted by the Au NCs. Albumin proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) are selected as the model proteins. Four small molecular drugs (e.g., ibuprofen, warfarin, phenytoin, and sulfanilamide) of different binding affinities to the albumin proteins are tested. It was found that the formation rate of fluorescent Au NCs inside the drug loaded albumin protein under denaturing conditions (*i.e.*, 60 °C or in the presence of urea) is slower than that formed in the pristine protein (without drugs). Moreover, the fluorescent intensity of the as-formed NCs is found to be inversely correlated to the binding affinities of these drugs to the albumin proteins. Particularly, the higher the drug-protein binding affinity, the slower the rate of Au NCs formation, and thus a lower fluorescence intensity of the resultant Au NCs is observed. The fluorescence intensity of the resultant Au NCs therefore provides a simple measure of the relative binding strength of different drugs tested. This method is also extendable to measure the specific drug-protein binding constant (K_D) by simply varying the drug content preloaded in the protein at a fixed protein concentration. The measured results match well with the values obtained using other prestige but more complicated methods.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53261/>

Introduction

Serum albumins such as human serum albumin (HSA) and bovine serum albumin (BSA) are the most abundant protein in plasma and play a vital role in maintaining the osmotic pressure of the blood compartment. They are also recognized as carrier proteins for small molecules of low water solubility, such as steroids, fatty acids, thyroid hormones, and a wide variety of drugs. The binding property (e.g., binding sites, binding affinity or strength) of these molecules to serum albumins forms an important topic in pharmacokinetics.¹⁻⁴ Several analytical methods have been developed to study the binding properties of different drugs to serum albumins, such as X-ray crystallography,^{5,6} nuclear magnetic resonance (NMR),⁷⁻¹¹ and surface plasmon resonance (SPR),^{12,13} etc. However, these methods are constrained by either a tedious and time-consuming analyzing process (e.g., growth of single crystal for X-ray crystallographic study), requirement of specialized and expensive equipment (SPR), or in need of costly isotope labeling (NMR) for detection. It is therefore highly desirable to develop alternative ways for small molecular drug screening in a fast, straight-forward, and cost-efficient manner.

Gold nanoclusters (Au NCs) are a special type of nanomaterial, which contain several to tens of metal atoms with sizes smaller than 2 nm.¹⁴⁻¹⁷ They have attracted extensive research interests due to their discrete and size-dependent electronic structure,^{18,19} and molecular-like absorptions and emissions.²⁰⁻²³ Such unique materials properties, in particular the strong fluorescence, have found diverse applications such as sensing and imaging in biological systems.²⁴⁻³² Ultrasmall fluorescent Au NCs can be synthesized using functional proteins, such as serum albumins, as template.³³ In a typical protein-templated synthesis of Au NCs, a certain amount of Au salts are first encapsulated inside the protein and subsequently reduced by the protein itself. The reducing ability of the protein is attributed to constituent functional amino acid residues (e.g., tyrosine) that can be activated by increasing the solution pH to alkaline. Unfolding of protein structure is considered as a critical step for the formation of Au NCs. This is because in an unfolded protein, more reducing functional groups can be exposed to the encapsulated Au salts. Protein unfolding can be achieved by heat treatment or exposure to denaturing agents. Introduction of small molecular drugs can also affect the unfolding process, *i.e.* modifying the midpoint denaturation temperature and the enthalpy of unfolding.^{34,35} The effect of all these factors, in turn can be reflected by the formation kinetics of fluorescent Au NCs and manifested in the fluorescence intensity of resultant Au NCs.³⁶

This video demonstrates the method of drug screening by synthesizing Au NCs in drug-loaded albumin proteins at a higher temperature (60 °C) or in the presence of denaturing agents (e.g., urea). The fluorescence intensity of resultant Au NCs is the signal readout. First, Au NCs are synthesized in HSA and BSA templates treated at 60 °C or in the presence of urea to show how protein unfolding (induced by heat treatment

or denaturants) affects the formation kinetics of Au NCs. Second, Au NCs are synthesized in protein templates preloaded with different drugs, and the drug loading effect on the relative fluorescence intensities of resultant Au NCs is studied, which provide the measure of relative binding strength. Finally, the Au NC-drug screening protocol is modified for quantitative measurement of drug-protein binding constant (K_D) by varying the drug content preloaded in the protein of a fixed concentration.

Protocol

Caution: Please consult the safety data sheets (SDS) of all involved chemicals before use. The drug screening experiment involves the synthesis and handling of nanomaterials, which may have additional hazards compared to their bulk counterpart. Please ensure all necessary control measures to be practiced throughout the experiment, including the use of engineering controls (fume hood) and personal protective equipment (PPE, e.g., safety length pants, closed-toe shoes, chemical resistant gloves, and safety goggles).

1. Preparation of Chemical Reagents for Drug Screening

1. Precursors for Au NCs synthesis
 1. Dissolve 30 mg of gold (III) chloride solution (99.99% trace metals basis, 30 wt.% in dilute HCl) in 6.9 ml of ultrapure water to prepare 15 mM of gold (III) chloride solution. Caution: gold chloride solution is corrosive and irritant. Wear proper PPE to avoid direct contact with eye and skin.
 2. Dissolve 74 mg of HSA or BSA in 1 ml of ultrapure water to prepare 74 mg/ml of protein stock solution.
2. Drug solutions
 1. Dissolve the desired amount of drug, e.g., 1.9 mg for (a) ibuprofen, 2.8 mg for (b) warfarin, 2.3 mg for (c) phenytoin, and 1.5 mg for (d) sulfanilamide in 20 μ l of DMSO to prepare 450 mM of drug stock solutions.
Note: DMSO is selected as the solvent because these drugs are hydrophobic and have poor solubility in water.
3. Other reagents
 1. Dissolve 600 mg of NaOH pellets in 10 ml of ultrapure water to prepare 1.5 M of NaOH solution. Dissolve 2.4 g of urea in 2 ml of ultrapure water to prepare 20 M of urea solution.

2. Synthesis of Protein-Templated Au NCs

1. HSA-templated Au NCs (HSA-Au NCs)
 1. Place two glass vials, each containing a micro magnetic stir bar in it, on two separate temperature controllable magnetic stirrers.
 2. Set the temperature of one magnetic stirrer to 60 °C, and label the glass vial on top of it as "60 °C"; while the other magnetic stirrer is kept at room temperature (RT) where the glass vial on top of it is labeled as "RT".
 3. Add 200 μ l of HSA solution, 200 μ l of ultrapure water, and 200 μ l of gold (III) chloride solution to each vial under constant stirring (set as 360 rpm unless otherwise specified) to allow the encapsulation of Au ions inside the protein template.
 4. 2 min later, add 20 μ l of NaOH solution to each vial so as to activate the reducing capability of HSA in forming Au NCs and start to record the reaction time as 0 min.
 5. Every 20 min, draw 50 μ l of solutions from each of the sample to a 384-well black plate and measure the emission spectrum with a microplate reader. The typical scanning setup: λ_{ex} = 370 nm, λ_{em} = 410 – 850 nm.
 6. Stop the magnetic stirrer after 100 min.
 7. Cool down the glass vials under running water in a sink. Caution: The glass vials are hot. Wear heat resistant gloves to avoid burning.
 8. Plot the photoemission spectra at all time for each sample to acquire the formation kinetics of Au NCs at different temperature conditions.
2. BSA-templated Au NCs (BSA-Au NCs)
 1. Place a glass vial containing a micro magnetic stir bar on the top of a magnetic stirrer. Leave the temperature as room temperature.
 2. Mix 200 μ l of BSA solution, 200 μ l of urea, and 200 μ l of gold (III) chloride solution in the glass vial under constant stirring.
 3. 2 min later, add 20 μ l of NaOH solution to activate the reducing capability of BSA to form Au NCs and start to record the reaction time as 0 min. Measure the photoemission spectrum of the reaction mixture hourly.
Note: The photoemission spectrum of BSA-Au NCs is measured less frequently because the formation rate of BSA-Au NCs is slower than that of HSA-Au at the same temperature due to less effectiveness of urea to unfold the protein.
 4. Stop the magnetic stirrer after 7 hr. Plot the photoemission spectra at all time to see the formation kinetics of Au NCs by urea denaturation.

3. Small Molecular Drug Screening

1. Screen relative binding affinity of different small molecular drugs to HSA
 1. Place five glass vials, each containing a magnetic stir bar in it, on top of a temperature controllable multipoint magnetic stirrer. Label these vials as a, b, c and d respectively for each drug, namely ibuprofen, warfarin, phenytoin, and sulfanilamide. Label the one with pure HSA as control.
 2. Add 200 μ l of HSA to each vial. Add 1 μ l of drug solution to the four corresponding vials. Add 1 μ l of DMSO to the control. Switch on the stirrer and set the spin speed to 360 rpm and incubate for 1 hr to allow the drug binding to HSA to complete.
 3. 1 hr later, add 200 μ l of Milli-Q water and 200 μ l of gold (III) chloride solution to each vial under constant stirring. Set the temperature to 60 °C under constant stirring for 10 min. Add 20 μ l of 1.5 M NaOH to each vial and start to record the reaction time as 0 min.

4. Quickly draw 50 μ l of solution from each vial to a 384-well black plate and measure the emission spectrum (results labeled as 0 min). Record the emission spectra of each sample every 10 min. Collect at least four spectra at four different time, that is 0, 10, 20, and 30 min.
 5. Repeat steps 3.1.1 - 3.1.4 several times to obtain results with a consistent trend. Stop the magnetic stirrer.
 6. Plot the time-resolved photoemission spectra for each sample (a - d, and control). Identify the peak intensity of all samples and plot against the time to compare the formation kinetics of Au NCs in different drug-loaded protein templates.
2. Measure the binding constant of a specific drug to HSA
1. Repeat steps 3.1.1 - 3.1.4. Replace the drug solution with ibuprofen solutions at four different concentrations (including a control sample using HSA only without drug loading). Label the glass vial according to the drug concentration correspondingly.
 2. 10 min later, cool down the glass vials under running water in a sink. Caution: The glass vials are hot. Wear heat resistant gloves to avoid burning.
 3. Draw 50 μ l of the above solutions from each vial to a 384-well black plate and measure the emission spectrum.
 4. Repeat steps 3.2.1 - 3.2.3 twice more to obtain another two sets of results at different drug concentrations.
 5. Stop the magnetic stirrer. Plot the photoemission spectra and analyze the results.
 1. For the raw data obtained in each individual batch, plot the photoemission spectra of each sample in software such as OriginPro software and find out the peak intensity.
 2. Calculate and plot the relative fluorescence intensity $[(I_0 - I) / I_0]$ against the drug concentration, where I and I_0 refer to the fluorescence intensity of Au NCs formed in drug-loaded HSA and pure HSA, respectively.
 3. Calculate the binding constant by fitting the data to a single site binding model using the Michaelis-Menten equation: $Y = R_{\max} \times C / (K_D + C)$, where R_{\max} indicates the maximum response signal, C is the concentration of ligand and K_D is the binding constant. Perform this in software such as OriginPro. Select the menu Analysis Fitting Nonlinear Curve Fit, then select Hill function from *Growth/Sigmoidal* category. On the Settings: Function Selection page, click *Fit* to show up the fitted results.

Representative Results

Protein unfolding is an important procedure for the formation of protein-templated Au NCs because more reactive functional groups (e.g., tyrosine residues) of a protein can be exposed to reduce the encapsulated Au ions and thus accelerate the formation rate of Au NCs. Heating and external denaturing agents are two common means to promote the protein unfolding process. **Figure 1** demonstrates the effect of heating and adding external denaturing agents on the formation kinetics of Au NCs, using HSA as a model protein. The effect of heating on the formation kinetics of Au NCs is first investigated by measuring the time course evolution of photoluminescence spectra (**Figure 1A and 1B**). When the reaction is carried out at 60 °C, red-emitting Au NCs are rapidly formed in a short period of time (100 min), as confirmed by the obvious peak centered at 670 nm of the photoemission spectra^{33,36} that increases progressively along with time (**Figure 1B**). As a contrast, no emission peaks of Au NCs at 670 nm are observed for reactions carried out at room temperature in the same period of time (**Figure 1A**).

The effect of introducing external denaturing agents on the formation kinetics of Au NCs is also investigated using BSA as template and urea as the denaturing agent. **Figure 1C** shows the time course evolution of photoluminescence spectra of the as-synthesized Au NCs. The similar emission peak of Au NCs at 670 nm is also observed, which suggests that BSA is also applicable for the synthesis of fluorescent Au NCs in the presence of external denaturing agent. However, the fluorescence intensity of resultant Au NCs is much lower than that formed at 60 °C even after a prolonged period of reaction time (7 hr). These results suggest that the formation of Au NCs in the presence of urea is much slower as compared to that formed at 60 °C due to the inefficiency of protein unfolding induced by an external denaturing agent at room temperature. Therefore, heating at 60 °C is selected as the preferred means of denaturation to screen for small molecular drugs in subsequent experiments with consideration of a rapid assay time.

Figure 2 shows the results of drug screening based on the formation kinetics of Au NCs with drug-loaded HSA. As shown in **Figure 2A**, the fluorescence intensity of Au NCs formed with the pure HSA template is consistently highest throughout the whole period of assay time (30 min), followed by those with sulfanilamide (d), phenytoin (c), warfarin (b), and ibuprofen (a) in sequence, which suggests that the formation rate of Au NCs in different templates follows the trend of control > d > c > b > a. Therefore, to shorten the assay time, only the photoemission spectra of all the resultant Au NCs at 10 min are compared to determine the relative binding affinity of drugs to HSA (**Figure 2B**). Multiple runs of experiment have been carried to confirm the consistency of this trend (**Figure 2C**). The spectrum intensity difference can be easily visualized from the digital photos of the resultant Au NCs as shown in **Figure 2C**(inset). The differential fluorescence intensities of the resultant Au NCs in the presence of different drug-loaded protein templates is inversely proportional to the binding strength of these drugs to HSA as determined in previous studies³⁶, which suggests that the binding of drugs improve the stability of protein against unfolding during the formation of Au NCs. Therefore, the binding affinities of these drugs to HSA can be easily differentiated by comparing the fluorescence intensity of as-formed Au NCs with drug-loaded HSA.

Finally, current drug screening method is applied to measure the binding constant of a specific drug to protein. Ibuprofen is selected to demonstrate on how to measure the binding constant of a small molecular drug to HSA. Ibuprofen solutions of different concentrations (0 – 450 μ M) are pre-incubated with HSA (74 mg/ml, 200 μ l) to form ibuprofen-HSA template before the synthesis of Au NCs. **Figure 3** shows the fluorescence spectra of the Au NCs formed in the HSA template pre-loaded with ibuprofen of different concentrations in a single batch at 10 min of reaction time. It can be seen that the fluorescence intensity decreases with the increasing amount of drug loaded to the protein template. To determine the binding constant of ibuprofen to the HSA protein, the relative fluorescence $(I_0 - I) / I_0$ of each Au NCs sample obtained from several different batches is calculated, where I_0 is the fluorescence intensity of the NC synthesized in pure HSA and I is the fluorescence intensity of the Au NCs synthesized in ibuprofen-loaded HSA (**Table 1**). The $(I_0 - I) / I_0$ values are plotted against the drug concentration (**Figure 4**, dotted line). The data is further fitted to a single site binding model using the Michaelis-Menten equation: $Y = R_{\max} \times C / (K_D + C)$, where R_{\max} indicates the maximum response signal, C is the concentration of ligand and K_D is the binding constant (**Figure 4**, solid line). This fitting gives a K_D value of ibuprofen to HSA of $0.74 \pm 0.1 \mu$ M. This value is very close to that ($0.5 \pm 1.0 \mu$ M) measured using NMR technique³⁷ thus further confirms the

reliability of current method to measure the binding constants of small molecular drug in homogeneous solutions, without using sophisticated equipment.

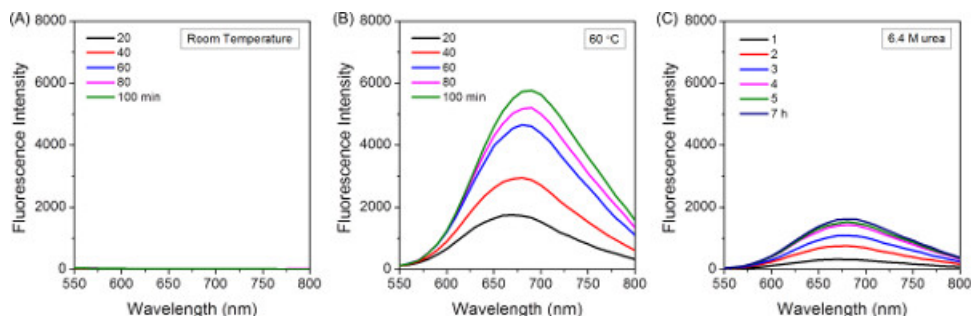


Figure 1. Effect of different denaturing conditions on the formation kinetics of Au NCs. Please click here to view a larger version of this figure.

Time resolved photoemission spectra of Au NCs formed in (A) HSA at room temperature, (B) HSA at 60 °C, and (C) BSA with 6.4 M urea.

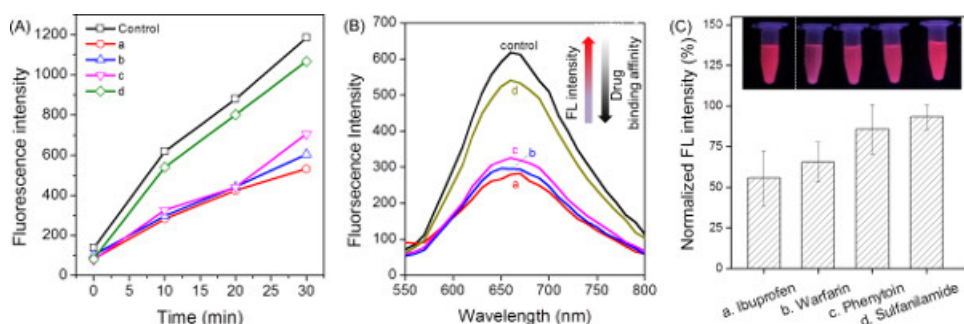


Figure 2. HSA-targeting drug screening based on fluorescence intensity of Au NCs formed in drug-loaded protein templates. Please click here to view a larger version of this figure.

(A) Formation kinetics of Au NCs formed in (a) ibuprofen-HSA, (b) warfarin-HSA, (c) phenytoin-HSA, (d) sulfanilamide-HSA, and pure HSA (control). (B) Photoemission spectra of HSA-Au NCs prepared at 60 °C in the presence of different drugs: (a) ibuprofen-HSA, (b) warfarin-HSA, (c) phenytoin-HSA, (d) sulfanilamide-HSA, and pure HSA (control). (C) Normalized photoemission intensity of HSA-drug-Au NCs against HSA-Au NCs (control). Both spectra and digital photos (inset of C) were taken at 10 min after addition of NaOH at 60 °C. ³⁶ Reproduced by permission of The Royal Society of Chemistry.

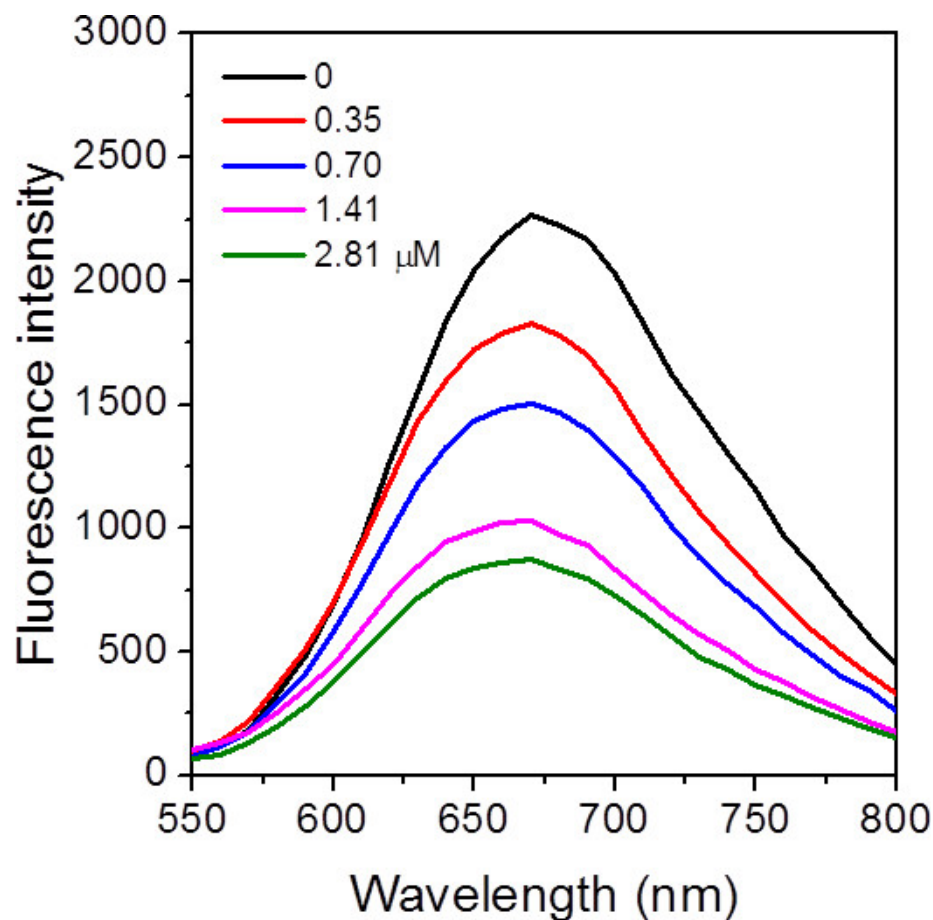


Figure 3. Concentration dependent study of drug loading on HSA protein.

Photoluminescence spectra of Au NCs formed in HSA loaded with ibuprofen at different concentrations in a single batch over 10 min of reaction time.³⁶ Adapted by permission of The Royal Society of Chemistry.

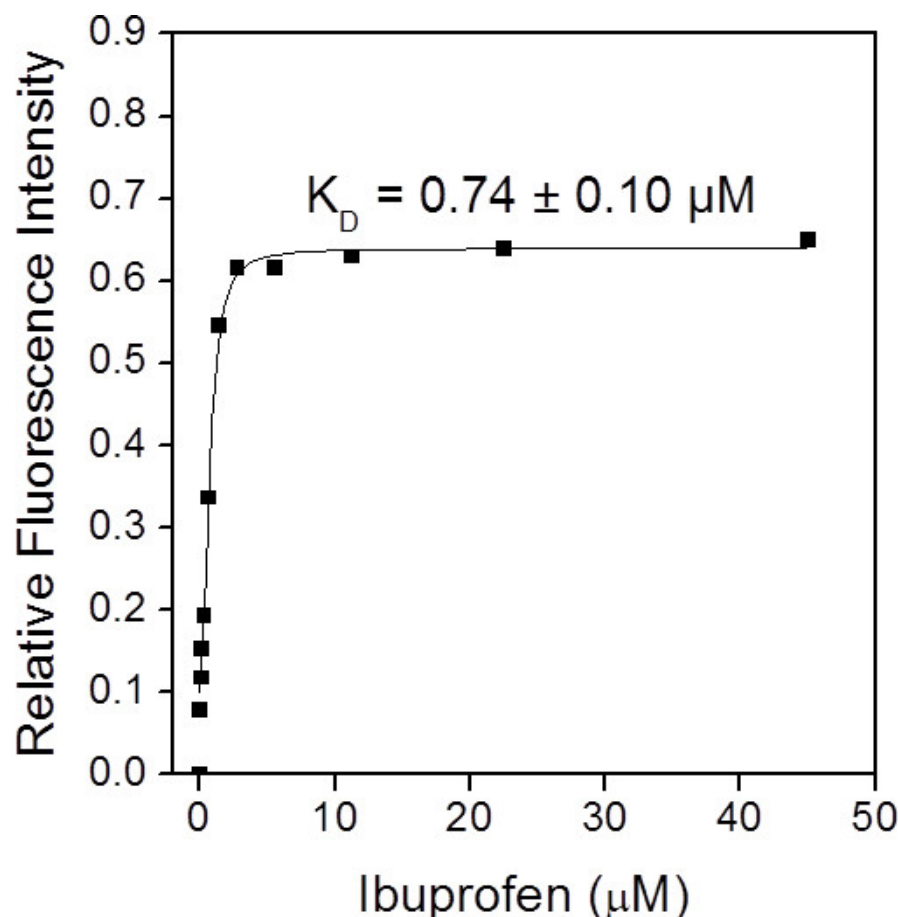


Figure 4. Determination of protein-drug binding constant from experimental data.

The K_D of ibuprofen binding to HSA was determined based on the relative fluorescence intensities of HSA-ibuprofen-Au NCs synthesized at 60 °C with increasing amount of drugs. The experimental data was fitted to a single site binding model using the Michaelis-Menten equation: $Y = R_{\text{max}} \times C / (K_D + C)$, where R_{max} indicates the maximum response signal, C is the concentration of ligand and K_D is the binding constant.³⁶ Reproduced by permission of The Royal Society of Chemistry.

Sample sequence number	Drug concentration (μM)	Fluorescence intensity (I)	Relative fluorescence intensity $[(I_0 - I)/I_0]$
1-1	0	2268 (I_0)	0
1-2	0.35	1829	0.19
1-3	0.70	1506	0.34
1-4	1.41	1033	0.54
1-5	2.81	872	0.62
2-1	0	1285 (I_0)	0
2-2	0.05	1186	0.08
2-3	0.09	1134	0.12
2-4	0.18	1089	0.15
3-1	0	1122 (I_0)	0
3-2	5.60	431	0.62
3-3	11.25	416	0.63
3-4	22.50	405	0.64
3-5	45.00	394	0.65

Table 1. Calculation of relative fluorescence intensities of Au NCs formed in protein templates with drug of varied concentrations.

Relative fluorescence intensities $[(I_0 - I)/I_0]$, I and I_0 refer to the fluorescence intensity of Au NCs formed in drug-loaded HSA and pure HSA, respectively) of Au NCs formed in HSA loaded with ibuprofen at different concentrations over 10 min of reaction time. Sample number x-y refers to the y^{th} sample prepared in the x^{th} batch.³⁶ Adapted by permission of The Royal Society of Chemistry.

Discussion

There are several critical steps that need to be highlighted in this method. In the protocol of screening the relative binding affinity of different small molecular drugs, Steps 3.1.2, 3.1.3, and 3.1.4 are critical to obtain good results showing consistent trend for the relative binding strength.

In these steps, the actions of adding chemicals and drawing reaction solutions for measurement should be as quickly as possible to minimize the time lag effect and the same sequence of adding chemicals and drawing reaction solutions for measurement should be practiced to ensure consistency. In the protocol of measuring the binding constant of ibuprofen to HSA, Step 3.2.4 is very important towards the success of K_D measurement. Though more concentration points can be selected, the time lag effect due to more samples to measure becomes more profound. To minimize the time lag effect, the synthesis at different concentrations can be separated into several different batches in parallel. In each batch, a control without drug should be included to ensure the consistency and eliminate the possible system errors.

In this video, HSA is selected as the model protein to direct the synthesis of fluorescent Au NCs for drug screening. Only four different drugs, i.e., (a) ibuprofen (b) warfarin, (c) phenytoin, and (d) sulfanilamide are tested here. As a matter of fact, the current method is generic and can be modified into several different forms. Theoretically, this method can be applied to other drugs, small molecules such as steroids, fatty acids, thyroid hormones, and stapled peptides, provided these molecules can bind to HSA and improve the stability of protein against unfolding to a different extent. Other proteins, not limited to albumins such as HSA and BSA as shown here, can also be used as the functional template for drug screening as long as they are able to direct the synthesis of fluorescent Au NCs (inclusive of active functional groups for Au NCs formation, such as tyrosine residues) without affecting drug binding sites. Even the choice of metal is flexible; other metal NCs such as Ag NCs can also be used as the fluorescence probe for drug screening.

In summary, current method is simple, fast and cost effective as compared to other more sophisticated techniques such as X-ray crystallography and NMR for studying protein-drug interactions. It requires no isotope labeling and allows direct fluorescent detection of protein-drug binding in homogenous solution. In this current work, we have demonstrated the concept using examples of drug binding to improve the protein stability. It might also be possible to extend the current method to screen drugs that destabilize proteins upon binding as an interesting topic for future study.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Y.N.T. would like to acknowledge the Agency for Science, Technology and Research (A*STAR), Singapore for the financial support under the JCO CDA grant 13302FG063.

References

- Flarakos, J., Morand, K. L., & Vouros, P. High-Throughput Solution-Based Medicinal Library Screening against Human Serum Albumin. *Anal. Chem.* **77**, 1345-1353, doi:10.1021/ac048685z (2005).
- Vuignier, K., Veuthey, J.-L., Carrupt, P.-A., & Schappler, J. Global Analytical Strategy to Measure Drug-Plasma Protein Interactions: From High-Throughput to In-Depth Analysis. *Drug Discov. Toda.* **18**, 1030-1034, doi:10.1016/j.drudis.2013.04.006 (2013).
- Zsila, F. Subdomain Ib Is The Third Major Drug Binding Region of Human Serum Albumin: Toward The Three-Sites Model. *Mol. Pharm.* **10**, 1668-1682, doi:10.1021/mp400027q (2013).
- Dalvit, C., Fagerness, P. E., Hadden, D. T. A., Sarver, R. W., & Stockman, B. J. Fluorine-NMR Experiments for High-Throughput Screening: Theoretical Aspects, Practical Considerations, and Range of Applicability. *J. Am. Chem. Soc.* **125**, 7696-7703, doi:10.1021/ja034646d (2003).
- Ghuman, J., Zunszain, P. A., Petitpas, I., Bhattacharya, A. A., Otagiri, M., & Curry, S. Structural Basis of the Drug-binding Specificity of Human Serum Albumin. *J. Mol. Biol.* **353**, 38-52, doi:10.1016/j.jmb.2005.07.075 (2005).
- Mao, H., Hajduk, P. J., Craig, R., Bell, R., Borre, T., & Fesik, S. W. Rational Design of Diflunisal Analogues with Reduced Affinity for Human Serum Albumin. *J. Am. Chem. Soc.* **123**, 10429-10435, doi:10.1021/ja015955b (2001).
- Dalvit, C. et al. High-Throughput NMR-Based Screening with Competition Binding Experiments. *J. Am. Chem. Soc.* **124**, 7702-7709, doi:10.1021/ja020174b (2002).
- Krenzel, E. S., Chen, Z., & Hamilton, J. A. Correspondence of Fatty Acid and Drug Binding Sites on Human Serum Albumin: A Two-Dimensional Nuclear Magnetic Resonance Study. *Biochemistr.* **52**, 1559-1567, doi:10.1021/bi301458b (2013).
- Lee, Y., Zeng, H., Ruedisser, S., Gossert, A. D., & Hilty, C. Nuclear Magnetic Resonance of Hyperpolarized Fluorine for Characterization of Protein-Ligand Interactions. *J. Am. Chem. Soc.* **134**, 17448-17451, doi:10.1021/ja308437h (2012).
- Salvi, N. et al. Boosting the Sensitivity of Ligand-Protein Screening by NMR of Long-Lived States. *J. Am. Chem. Soc.* **134**, 11076-11079, doi:10.1021/ja303301w (2012).
- Zsila, F. Circular Dichroism Spectroscopic Detection of Ligand Binding Induced Subdomain IB Specific Structural Adjustment of Human Serum Albumin. *J. Phys. Chem.* **117**, 10798-10806, doi:10.1021/jp4067108 (2013).
- Navratilova, I., & Hopkins, A. L. Fragment Screening by Surface Plasmon Resonance. *ACS Med. Chem. Lett.* **1**, 44-48, doi:10.1021/ml900002k (2010).
- Wang, Y. et al. Investigation of Phase SPR Biosensor for Efficient Targeted Drug Screening with High Sensitivity and Stability. *Sensor. Actuat. B-Chem.* **209**, 313-322, doi:10.1016/j.snb.2014.11.134 (2015).
- Lu, Y., & Chen, W. Sub-Nanometre Sized Metal Clusters: From Synthetic Challenges to The Unique Property Discoveries. *Chem. Soc. Rev.* **41**, 3594-3623, doi:10.1039/C2CS15325D (2012).
- Yu, Y., Yao, Q., Luo, Z., Yuan, X., Lee, J. Y., & Xie, J. Precursor Engineering and Controlled Conversion for The Synthesis of Monodisperse Thiolate-Protected Metal Nanoclusters. *Nanoscal.* **5**, 4606-4620, doi:10.1039/C3NR00464C (2013).
- Jin, R. Quantum Sized, Thiolate-Protected Gold Nanoclusters. *Nanoscal.* **2**, 343-362 (2010).
- Jiang, D.-e. The Expanding Universe of Thiolated Gold Nanoclusters and Beyond. *Nanoscal.* **5**, 7149-7160, doi:10.1039/C3NR34192E (2013).
- Aikens, C. M. Electronic Structure of Ligand-Passivated Gold and Silver Nanoclusters. *J. Phys. Chem. Lett.* **2**, 99-104, doi:10.1021/jz101499g (2010).

19. Gao, Y., Shao, N., Pei, Y., Chen, Z., & Zeng, X. C. Catalytic Activities of Subnanometer Gold Clusters (Au_{16} – Au_{18} , Au_{20} , and Au_{27} – Au_{35}) for CO Oxidation. *ACS Nan.* **5**, 7818–7829, doi:10.1021/nn201817b (2011).
20. Negishi, Y., Nobusada, K., & Tsukuda, T. Glutathione-Protected Gold Clusters Revisited: Bridging the Gap between Gold(I)–Thiolate Complexes and Thiolate-Protected Gold Nanocrystals. *J. Am. Chem. Soc.* **127**, 5261–5270, doi:10.1021/ja042218h (2005).
21. Zhu, M., Aikens, C. M., Hollander, F. J., Schatz, G. C., & Jin, R. Correlating the Crystal Structure of A Thiol-Protected Au_{25} Cluster and Optical Properties. *J. Am. Chem. Soc.* **130**, 5883–5885, doi:10.1021/ja801173r (2008).
22. Yu, Y. *et al.* Identification of a Highly Luminescent $\text{Au}_{22}(\text{SG})_{18}$ Nanocluster. *J. Am. Chem. Soc.* **136**, 1246–1249, doi:10.1021/ja411643u (2014).
23. Jiang, J. *et al.* Oxidation at the Core–Ligand Interface of Au Lipoic Acid Nanoclusters That Enhances the Near-IR Luminescence. *J. Phys. Chem.* **118**, 20680–20687, doi:10.1021/jp5060217 (2014).
24. Zhu, Y., Qian, H., & Jin, R. An Atomic-Level Strategy for Unraveling Gold Nanocatalysis from the Perspective of $\text{Au}_n(\text{SR})_m$ Nanoclusters. *Chem. Eur. J.* **16**, 11455–11462, doi:10.1002/chem.201001086 (2010).
25. Niesen, B., & Rand, B. P. Thin Film Metal Nanocluster Light-Emitting Devices. *Adv. Mater.* **26**, 1446–1449, doi:10.1002/adma.201304725 (2014).
26. Shang, L., Dong, S. J., & Nienhaus, G. U. Ultra-Small Fluorescent Metal Nanoclusters: Synthesis and Biological Applications. *Nano Toda.* **6**, 401–418, doi:DOI 10.1016/j.nantod.2011.06.004 (2011).
27. Wu, X., He, X., Wang, K., Xie, C., Zhou, B., & Qing, Z. Ultrasmall Near-Infrared Gold Nanoclusters for Tumor Fluorescence Imaging in Vivo. *Nanoscal.* **2**, 2244–2249 (2010).
28. Archana, R. *et al.* Molecular-Receptor-Specific, Non-Toxic, Near-Infrared-Emitting Au Cluster-Protein Nanoconjugates for Targeted Cancer Imaging. *Nanotechnolog.* **21**, 055103 (2010).
29. Yue, Y., Liu, T. Y., Li, H. W., Liu, Z. Y., & Wu, Y. Q. Microwave-Assisted Synthesis of BSA-Protected Small Gold Nanoclusters and Their Fluorescence-Enhanced Sensing of Silver(I) Ions. *Nanoscal.* **4**, 2251–2254, doi:Doi 10.1039/C2nr12056a (2012).
30. Liu, Y., Ai, K., Cheng, X., Huo, L., & Lu, L. Gold Nanocluster Based Fluorescent Sensors for Highly Sensitive and Selective Detection of Cyanide in Water. *Adv. Funct. Mater.* **20**, 951–1907, doi:10.1002/adfm.200902062 (2010).
31. Liu, J., Yu, M., Zhou, C., Yang, S., Ning, X., & Zheng, J. Passive Tumor Targeting of Renal-Clearable Luminescent Gold Nanoparticles: Long Tumor Retention and Fast Normal Tissue Clearance. *J. Am. Chem. Soc.* **135**, 4978–4981, doi:10.1021/ja401612x (2013).
32. Negishi, Y. *et al.* Controlled Loading of Small Au_n Clusters ($n = 10$ – 39) onto $\text{BaLa}_4\text{Ti}_4\text{O}_{15}$ Photocatalysts: Toward an Understanding of Size Effect of Co-Catalyst on Water Splitting Photocatalytic Activity. *J. Phys. Chem. C*, doi:10.1021/jp5122432 (2015).
33. Xie, J., Zheng, Y., & Ying, J. Y. Protein-Directed Synthesis of Highly Fluorescent Gold Nanoclusters. *J. Am. Chem. Soc.* **131**, 888–889, doi:10.1021/ja806804u (2009).
34. Celej, M. S., Montich, G. G., & Fidelio, G. D. Protein Stability Induced by Ligand Binding Correlates with Changes in Protein Flexibility. *Protein Sci.* **12**, 1496–1506, doi:10.1110/ps.0240003 (2003).
35. Layton, C. J., & Hellinga, H. W. Thermodynamic Analysis of Ligand-Induced Changes in Protein Thermal Unfolding Applied to High-Throughput Determination of Ligand Affinities with Extrinsic Fluorescent Dyes. *Biochemistr.* **49**, 10831–10841, doi:10.1021/bi101414z (2010).
36. Yu, Y., New, S. Y., Xie, J., Su, X., & Tan, Y. N. Protein-Based Fluorescent Metal Nanoclusters for Small Molecular Drug Screening. *Chem. Commun.* **50**, 13805–13808, doi:10.1039/C4CC06914E (2014).
37. Shortridge, M. D. *Nuclear Magnetic Resonance Affinity Screening Methods for Functional Annotation of Proteins and Drug Discover.* Ph.D. thesis, The University of Nebraska - Lincoln, (2010).