FRET-Like Fluorophore-Nanoparticle Complex for Highly Specific Cancer Localization

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Abstract Fluorophore mediated bio-signal retrieval has been extensively used in molecular imaging. However, only a limited number of fluorophores can be used for humans and their quantum yield is usually low. Another important issue is emitting fluorescence at the disease site, with a minimal non-specific emission at any other sites. Artificial quenching and enhancing of fluorescence was found to be possible by manipulating the distance between a fluorophore and a certain type of nanometal particle. Utilizing this unique property, we have designed a novel, FRET-like, fluorophore-nanoparticle complex. The complex emits fluorescence conditionally only at a disease site at an enhanced level. As a model system, our complex is designed to target breast cancer. As an initial step for developing this cancer locator, fluorescence alteration was studied when a spacer at various lengths is placed between a nanogold particle and a safe fluorophore.

1 Introduction

Fluoropohore mediated, molecular imaging is highly beneficial for understanding mechanisms of various biomolecular functions. For in vivo bioimaging, such as cancer detection or diagnosis, the fluorohpore must be non-toxic. Fluorophores with excitation and emission in a near infrared (NIR) range have advantages over those in a visible range. They can be better differentiated from the naturally occurring fluorescence in tissue and NIR penetrates deeper into tissue [1, 2]. Fluorophores with high quantum yield is highly desirable, especially for detecting small and/or deeply seated tumors. In order for a molecular imaging to be highly effective, the fluorescence contrast should be maximized at the tumor site, with a minimal non-specific emission. A smart

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approach is, therefore, using an entity with conditional emission, similar to Förster resonance energy transfer (FRET).

Since fluorophores emit fluorescence when their electrons are excited by photons, their fluorescence may be altered when they are placed within a strong surface plasmon polarion field (SPPF) of nano sized metal particles (NMPs). Gold, silver, and platinum are exemplary metals possessing strong SPPF [3]. When the NMP's electrons couple with excited electrons of a fluorophore, an instantaneous electron attraction occurs, altering the energy states of the electrons involved in fluorescence emission. The level of this alteration depends upon the strength of the SPPF where the fluorophore is placed, and the field strength depends upon the metal type, NMP size, and distance from the NMP surface [4, 5, 6, 7, 8]. If a fluorophore is placed within a very strong SPPF, most electrons of the fluorophore, including the ones for fluorescence emission, are attracted to the NMP, resulting in fluorescence quenching. If the fluorophore is at a particular distance from the NMP, and attracts the electrons participating in self-quenching, then the fluorescence is enhanced.

This interesting property of NMPs on a fluorophore can be effectively utilized for artificial fluorescence alternation. Utilizing nanogold particles (NGPs), we are currently developing a FRET-like, highly breast cancer specific, fluorescent contrast agent. The design of this novel agent is as follows: the NGP surface is treated with hydrophilic bio-polymer, and conjugated with cancer targeting molecules. The fluorescence of the fluorophore is manipulated by placing double spacers (one long and one short) between the fluorophore and NGP, and it is specially designed to be conditionally emitted only at a particular tumor site, at an enhanced fluorescence level.

Indocyanine Green (ICG, MW 775) is one of a few FDA-approved fluorophores. Its excitation and emission peaks are at 780 and 830 nm (both in NIR), respectively. Its quantum yield (QY) is only 0.0028 in the saline buffer and 0.012 in blood [9]. ICG does not have functional group for conjugation. Achilefu's group has developed an ICG derivative, Cypate, with two carboxylic acid groups [10].

As an initial step for developing this novel cancer locator, quenching and enhancement of Cypate fluorescence by NGPs were studied, and the result is presented here.

2 Materials, Instruments and Methods

Nanogold colloids (NGPs; 5 and 10 nm in citric and tannic acids) and Protein A conjugated NGP (NGP-PA) were from Ted Pella (Redding, CA, USA). L-glutathione (LG; Sigma/Aldrich; St. Louis, MO, USA) was immobilized on the NGP surface by the protocol developed by our research group [7, 8].

Cypate was covalently immobilized on the surface of the NGPs via LG (NGP-LG) or PA (NGP-PA) using the protocol established by our group [6].

N,N' Dicyclohexylcarbodiimide (DCC; Sigma) was used to catalyze the reaction. First, dissolve Cypate and DCC separately in a minimal amount of ethanol (< 50 μl). Mix the Cypate and DCC solutions at a molar ratio of 1:12, and immediately transfer them to NGP-LG or NGP-PA solution. Stir the mixture of Cypate and NGP-LG at room temperature for 30 min. For Cypate and NGP-PA, stir at 4°C for 5 h.

All samples (Cypate with and without NGP) were in a 0.001 M PBS, and Cypate concentration was 30 μM, at which Cypate has maxium fluorescence [11]. Fluorescence of samples was measured in 96-well EIA/RIA plate (Corning, New York, USA) using a fluorometer (Spectra Gemini XPS; Molecular Devices Corp.; Sunnyvale, CA, USA). The excitation and emission wavelengths used in our study were 780 and 830 nm, respectively.

3 Results and Discussion

3.1 Free Cypate Fluorescence by Free NGP Colloids

First, the fluorescence alteration of free Cypate by free NGPs (surfactant, tannic acid, MW = 1516) by NGPs was studied. This study result may be valuable also for fluorophore mediated biosensing [6, 7, 8] 5 and 10 nm NGPs at a weight (wt) concentration range of 0∼60 μg/ml were applied to 30 μM Cypate solution, and the fluorescence was observed (Fig. 1). It should be noted that NGP molar concentration in this range is much less than Cypate concentration (30 μM), i.e., NGP concentration is the limiting condition.

As can be seen in the figure, for both NGP sizes, at concentrations greater than 6 μg/ml, the fluorescence was quenched, and the quenching level increased with the increase in NGP concentration. At the same wt concentration, 5 nm NGPs showed greater quenching than those at 10 nm, probably because the

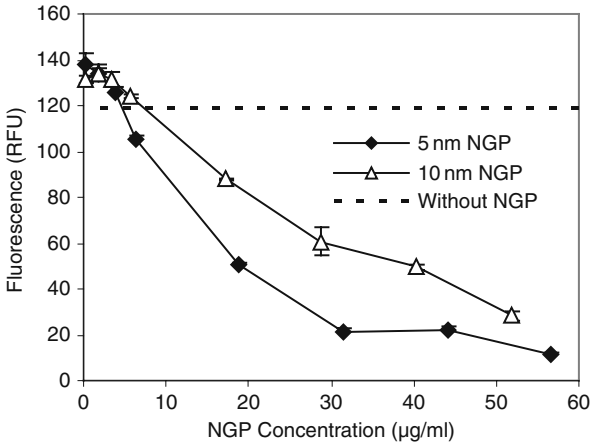

Fig. 1 Fluorescence of free Cypate with various concentrations of free NGP

number of 5 nm NGPs is 8 times greater than that of 10 nm NGP, and the total surface area is about 2 times larger. Also, due to its smaller size, 5 nm NGPs should have much great mobility than that of 10 nm, resulting in greater probability for Cypate to be near the NGP.

At very low concentrations (< 6 μg/ml), the NGPs enhanced the fluorescence. This phenomenon may be explained by Fig. 2. For free NGPs and fluorophores, when the fluorophore concentration is fixed, the distances between NGP and fluorophore is determined by NGP concentration. Cypate is negatively charged in 0.001 M PBS. NGPs are positively charged and negatively charged citric and, therefore, tannic acid ions are adsorbed on their surface. At a higher NGP concentration [Fig. 2(a)-three NGPs], the distance between NGP and Cypate is shorter and, thus, more Cypate molecules are near the NGP surface, resulting in more fluorescence quenching. When the NGP concentration is very low [Fig. 2(b)-one NGP], the average distance between Cypate and NGP is longer, and the fluorescnece is either slightly enhanced or unaffected.

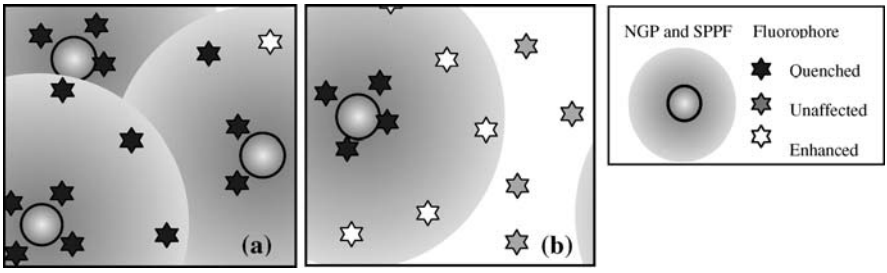

Fig. 2 Schematic diagram for fluorescence of free Cypate with (**a**) high and (**b**) low NGP concentration in solution

3.2 Fluorescence Change by NGP-Short Spacer

The next was to study the fluorescence when Cypate was conjugated onto NGP surface (e.g. in bioimaging). LG (MW = 307) was used as the short spacer, and its length was previously estimated to be 1 nm by our group [7, 8]. The fluorescence was observed when Cypate was conjugated to 5 and 10 nm NGPs via LG, at an NGP concentration range of 0∼60 μg/ml (Fig. 3).

At all concentrations, the fluorescence decreased. In general, for higher concentration of NGP, more fluorescence was quenched. At the same wt concentration, 5 nm NGPs have binding sites (LG molecule numbers) twice that of 10 nm NGPs. However, they quenched fluorescence less, probably because of the difference in SPPF strength for the different NGP size (i.e., higher field strength for a larger particle size).

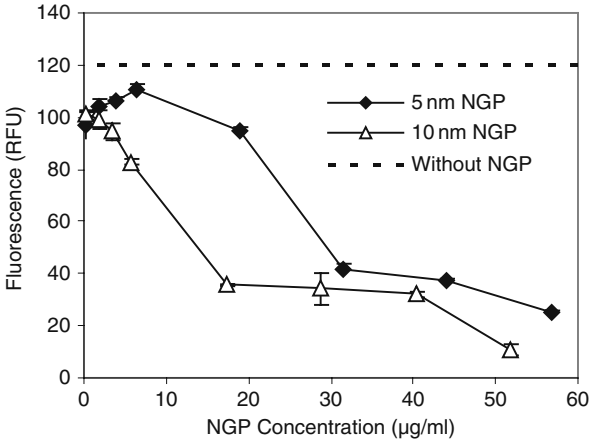

Fig. 3 Cypate fluorescence when Cypate was conjugated to NGP-LG at various concentrations

For 5 nm NGPs, at a very low concentration (< 6 μg/ml), the quenching effect did not increase with the increase in NGP concentration, probably because only a portion of Cypate was conjugated to NGPs and most of Cypate was present in a free form in the solution.

3.3 Fluorescence Change by NGP-Longer Spacer

To study the fluorescence change by NGP with a longer spacer, Protein A (PA; MW 40∼60 kD) was used as the spacer (Fig. 4). Since PA is a globular protein and we did not know how the protein is conjugated on the NGP surface, it was difficult to estimate its length as a spacer, although one of our simulation results estimates it to be approximately 5 nm (not published). The ratios of PA to NGP

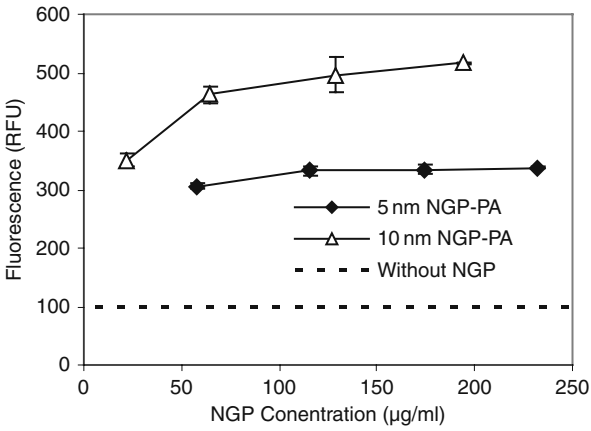

Fig. 4 Cypate fluorescence when Cypate was conjugated to NGP-PA at various concentrations

were 4:1 and 16:1 for 5 and 10 nm NGPs, respectively. NGPs at a concentration range of 20∼250 μg/ml were applied to Cypate solution (Fig. 4).

The fluorescence of Cypate was enhanced extensively by both 5 and 10 nm NGPs. The enhancement was greater for a higher NGP concentration, especially for 10 nm NGPs. At the same weight concentration range, a 5 nm NGP has binding sites (PA molecule numbers) 2 times more than a 10 nm NGP does. However, it enhances fluorescence less than 10 nm NPG does, confirming the difference in the SPPF strength for different sized particles.

4 Conclusions

Utilizing the SPPF of NMPs, artificial fluorescence alteration of Cypate (NIR fluorophore) was achieved. With manipulating the distance between an NGP and a fluorophore by placing a molecular spacer between them, upto 90% percent of fluorescence quenching and 500% of enhancement were achieved. The level of quenching and enhancement are currently being optimized to develop a novel, FRET-like, fluorophore-nanoparticle complex for higly specific breast cancer localization.

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