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Quantification of Cardiac Biomarkers using Label-free and Multiplexed Gold Nanorod Bioprobes for Myocardial Infarction Diagnosis

Liang Tang* and Justin Casas

Department of Biomedical Engineering, University of Texas at San Antonio, San Antonio, TX 78249, U.S.A

Abstract

Gold nanorod (GNR) is an attractive optical transducer for label-free biosensing owing to the localized surface plasmon resonance (LSPR) which is highly sensitive to the dielectric constant of the surrounding medium modulated by biological bindings. By adjusting the nanorod aspect ratio (length to width ratio), desired absorption wavelength can be continuously tuned from 600 to 1,100 nm. Here we demonstrated a linear relationship between the aspect ratio and the LSPR peak wavelength. Taking advantage of this tunability feature, we developed a multiplexed GNR sensor by combining nanorods with distinct LSPR wavelengths. Specifically, GNRs of AR 2.1 and 4.2 exhibiting longitudinal plasmonic band of 640 and 830 nm respectively were functionalized with specific antibody. Concentrations of multiple analytes were measured by correlating to the spectral shift at the distinct plasmon band maxima upon specific binding. The practical use of this mixed bioprobes for simultaneous quantification of cardiac biomarkers (myoglobin and cardiac troponin I) in the clinically significant sensing range was described. The LSPR red shift magnitude is linearly proportional to the increase in the target analyte concentration ($R^2 = 0.98$). The calibration curve can clearly differentiate varying biomarker amounts with a high specificity. For multiplexed biosensing, the plasmon shift at the dedicated peak wavelength can be specifically correlated with spiked biomarker for simultaneous detection in the sample mixture. This technology can be further transformed onto miniaturized biochips based on the nanosized optical transducer to allow point-of-care blood testing for risk stratifications of cardiac patients in clinical settings.

Keywords

Gold nanorod; Multiplex biosensing; localized surface plasmon resonance; Cardiac biomarker; Myocardial infarction diagnostics

Introduction

Myocardial infarction (MI) is a major cause of death worldwide (Thygesen et al., 2012). However, the survival rate for the U.S. patients hospitalized with MI is approximately 90%

to 95%. This suggests the paramount importance of early diagnosis to deliver prime-time medical response for a significant improvement in survival. Identifying a patient who is currently experiencing a MI can be straightforward. When the patients with chest pain are presented in emergency room, a blood draw is routinely performed to detect rise in the biomarkers concentration due to myocardial cell damage. The preferred biomarker for MI diagnosis is cardiac troponin I (cTnI), which has nearly absolute cardiac specificity as well as high clinical sensitivity to reflect even microscopic zones of myocardial necrosis (Jaffe et al., 2000; Liu et al., 2006; Tsutamoto et al., 2010; Venge et al., 2009). Elevated cTnI levels may be detected in patient's blood 3~6 hours after the onset of a chest pain, reaching peak level within 16~30 hrs. Thus, the refined MI diagnostic criteria set forth by the American College of Cardiology (ACC) and European Society of Cardiology (ESC) gave more prominence to blood testing of biomarkers. According to the new guidelines, a cTnI rise in blood testing accompanied by either typical symptoms, ST elevation or depression in electrocardiogram (ECG) tracings or coronary intervention are diagnostic of MI (Alpert et al., 2000). Additionally, to achieve an early and specific cardiac diagnosis, combination of two or more cardiac biomarker detection is widely used in clinical practice. Based on a recent clinical study, a combination of cTnI and myoglobin (MG) can achieve a diagnostic sensitivity of 97% and a 99% negative predictive value within 90 mins of ED presentation. (McCord et al., 2001) Myoglobin is a protein that transports oxygen in muscle tissues. As early as one hour following the onset of chest pain, when cTnI is still in the normal range in bloodstream, myoglobin levels may already be elevated. Thus, a multi-biomarker detection strategy can significantly improve the reliability of cardiovascular diagnosis at early stage.

Although ECG and echocardiogram can be easily obtained within minutes at point of patient care, blood testing in current clinical practice represents a challenge and bottleneck for rapid, prime-time MI triage. This is because of the time delay caused by the logistics of sending blood samples to hospital central lab for laborious analysis by benchtop methodologies including ELISA, HPLC, and PCR. Therefore, it is highly desirable to develop a portable biosensor to perform at point of patient care. Nanobiotechnology offers an attractive route for highly sensitive immunoassays that require small amounts of sample in a miniaturized format. Specifically, gold nanoparticles (NPs) have been extensively utilized for biosensing (Dreaden et al., 2011; Marinakos et al., 2007; Nath and Chilkoti, 2002). The optical transduction of biological binding events is based upon the phenomenon of localized surface plasmon resonance (LSPR). (Jain et al., 2006a; Hutter and Fendler, 2004) According to the plasmon hybridization mode (Prodan et al., 2003), the plasmon resonance frequency is largely determined by the geometry of the colloidal metal structure, as a result of electrostatic interactions between confined electrons distributed over the surfaces of the metal conductor. Thus, anisotropic nanostructures usually exhibit superior optical properties than spherical NPs. (Zeman, 2011; Malinsky, 2001; Sun and Xia, 2002; Lee and El Sayed, 2006) Gold nanorods has been at the center of recent studies and applications based on wavelength shifts due to changes in dielectric properties in the vicinity of the functionalized nanorods have been reported (Mayer and Hafner, 2011; Petryayeva and Krull, 2011; Jain et al., 2006a; Chen et al., 2007; Marinakos et al., 2007; Tang et al., 2013). Compared to conventional methods (Tang et al., 2006), LSPR eliminates detection tags such as fluorophore, enzyme, and radioactive agents, thereby enabling label-free detection

mechanism. Since protein determination can lead to arduous processes each with their own limits, a gold nanorod detection scheme could serve to speed up and decrease the cost of biomarker detection.

The sensitivity as well as the tunability of the resonance wavelength maximum is closely related to geometrical parameters, especially aspect ratio (length to width ratio). (Sonnichsen et al., 2002; Link and El Sayed, 1999) As such, the longitudinal absorption wavelength can be selectively tuned from the visible to the near-infrared (NIR) region as aspect ratio increases. This size- and shape-dependent optical property provides an unique opportunity to develop a multiplexed nano-biosensing by combining variable GNRs of distinct plasmonic wavelengths. (Huang et al., 2009; Yu and Irudayaraj, 2007) Yu et al. described the first multiplex LSPR detection scheme using nanorods of varying aspect ratios. (Yu and Irudayaraj, 2007) The study has shown that concentrations of three IgGs can be measured simultaneously correlating to red-shifts at distinct plasmon absorption spectrum. Although it is a very promising technique in the buffered model system, it is largely unknown about the sensing performance for real detection of disease-representing biomarkers in the clinically meaningful range. Herein, we developed a multiplexed GNR nanosensor to simultaneously detect cTnI and MG at various concentrations. This study is of high clinical significance that could aid in rapid acute MI diagnosis in clinical settings. To our knowledge, our study is the first demonstration of the multiplex nanorod based biosensor application for these two clinically relevant cardiac biomarkers. This simple, label-free nanosensor provides a new paradigm in innovative solutions to the ever-increasing clinical demand of point-of-care bioanalytical devices.

Materials and Methods

Materials

Hydrogen tetrachloroaurate trihydrate (HAuCl_4 ; 99%), sodium borohydride (NaBH_4 ; 99%), Cetyltrimethylammoniumbromide (CTAB), L-ascorbic acid (AA), silver nitrate (AgNO_3 ; 99%), dodecanthiol (DDT), 11-mercaptoundecanoic acid (MUDA; >95%), N-hydroxysulfosuccinimide (Sulfo-NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC), human plasma, and fibrinogen were obtained from Sigma-Aldrich (St. Louis, MO). Highly purified human cardiac troponin I (cTnI), myoglobin (MG), and their respective murine monoclonal antibodies were from Fitzgerald Industries (Acton, MA).

Biofunctionalization of Au nanorods for biological probe

Gold nanorods (GNRs) were chemically synthesized using seed mediated growth method (Casas et al., 2013). Longitudinal plasmonic wavelength of the fabricated GNRs was tunable from 600-1,050 nm by varying the nanoparticle aspect ratio (length to width ratio). UV-vis spectrophotometer and scanning electron microscopy (Hitachi S5500) were used to characterize the size (length, width and aspect ratio), shape, and absorption spectra of the nanorods.

To construct a functional biosensor, the CTAB bilayer on nanorod surfaces were effectively replaced by alkanethiols (e.g. MUDA) to expose carboxyl groups, followed by immobilization of biological receptors as previously reported.(Casas et al., 2013) Briefly, 4 mL DDT solution was mixed with 2 ml GNR concentrate. After gentle shake, 8 mL acetone was added, resulting in a clear separation of the aqueous (lower) and organic (upper) layer where DDT-coated nanorods were present. Then, 2 ml toluene and 9 ml methanol were added to the top layer, followed by centrifuge (5,000 rpm for 15 minutes) into a pellet. The pellet was resuspended in 2 mL toluene via sonication. This suspension was then added under vigorous stirring to 9 mL MUDA (0.01M in toluene) at 70°C. After 15 minutes the solution was allowed to cool down to room temperature as the nanoparticles settled to the bottom. The aggregates were finally redispersed in 1 ml of Tris-borate EDTA buffer. Following these procedures, the densely-packed CTAB bilayer was replaced by alkanethiols to fully activate the GNR surfaces with carboxyl groups. The COOH-terminated nanorods can then be effectively immobilized with antibodies for cTnI and MG respectively through EDC/sulfo-NHS bioconjugate chemistry.

Label free, multiplexed plasmonic assay based on GNR bioprobes

Nanorods of different size which manifest distinct longitudinal SPR band maxima (e.g. 640 nm for MG and 830 nm for cTnI assay) were immobilized with antibodies for cTnI and MG, respectively. Afterwards, spiked samples containing various cTnI and/or MG concentrations in the sensing range were incubated with functional GNR bioprobes as prepared above for 30 minutes under mild agitation (500 rpm shaker). Upon biological binding of cTnI and MG with their respective antibodies on the nanorod surface, the resulting spectral shift in the longitudinal peak wavelength was a function of the antigen concentration in the sample. A pronounced red shift at 640 nm represents a detection of myoglobin, while plasmon shift at 830 nm corresponds to cTnI binding. The specificity of this label-free plasmonic nanosensor was investigated by probing with various irrelevant proteins and the resulting plasmon responses respectively.

Results and Discussion

Gold nanorods of varying aspect ratio were fabricated by fine tuning the optimal chemical reaction parameters. TEM imaging of the nanoparticles confirmed monodispersion, narrow size distribution, and yield of 93.5–98.5%. It has been reported that the tunability of the peak resonance wave is closely related to the length to width ratio (Liz-Marzan, 2006). We found a linear correlation between the aspect ratio and the longitudinal plasmon band maxima (λ_{max}) in the following equation.

$$\lambda_{max}=90.7R+448.3 \quad (1)$$

where R is the nanorod aspect ratio. As shown in Fig. 1A, increasing aspect ratio resulted in a longer LSPR absorption, exhibiting a plasmonic peak from 600 to 1,100 nm. This experimental data corroborates the theoretical calculations by the El-Sayed group.(Jain et al., 2006b) Fig. 1B shows the absorption spectra of these variable sized GNRs. The longitudinal band is a stronger band corresponding to electron oscillation along the long axis

of the nanorod. While the transverse band is around 520 nm which is the characteristic absorption for gold element, the longitudinal band is highly tunable from visible to NIR region. For the present study, this feature provides the material basis to design the subsequent multiplex biosensing scheme, which will be discussed below.

Label-free plasmonic nanosensor for myoglobin detection

Before simultaneous detection, individual nanosensor for single analyte detection was developed. The sensing performance of the cTnI sensor has been extensively studied in our prior work. (Tang et al., 2013; Casas et al., 2013) Herein, the myoglobin (MG) sensor was investigated in terms of sensitivity and specificity. A series of spiked MG sample at clinically significant concentrations up to 300 ng/ml was probed by GNR sensor on which anti-MG molecules were immobilized. The sensitivity to the refractive index change due to specific binding at the rod surface can provide efficient optical transduction of interfacial binding events that can be effectively exploited to develop a label free detection. In practice, UV-vis spectroscopy was used to monitor the red-shift in plasmon band maxima. Since longitudinal SPR is much more sensitive to local refractive index change, longitudinal spectral shift was focused for this label-free plasmonic biosensing.

Upon binding of myoglobin with its specific antibody molecules immobilized on the GNR sensor, this event perturbed the refractive index immediately surrounding the nanorods. As such, a pronounced red shift of ca. 14 nm in the longitudinal plasmon peak was observed for 50 ng/ml MG sample. Increase in the MG concentration caused a larger spectral shift as expected, which demonstrated a linear relationship between the shift magnitude and the target concentration (Fig. 2A). The increase rate of the plasmon shift was reduced at higher concentrations due to saturation. The upper limit of a reliable detection was determined to be 400 ng/ml, above which the shift was almost a flat line in the plot. Nevertheless, within the targeted clinical sensing range, The spectral sensitivity, defined as relative shift in resonance wavelength with respect to the refractive index change of surrounding medium, is satisfactory so that the standard curve is capable of clearly differentiating MG amounts for accurate assay. Besides sensitivity, the antibody effectively functionalized on the GNR sensor guarantees the specificity for myoglobin detection. Three control samples (cTnI, fibrinogen, and human plasma) were chosen to compare their SPR responses when probed by the MG nanosensor. Fig. 2B shows the spectral shifts caused by the nonspecific adsorption of control proteins on the anti-MG modified nanorods. All the plasmon shifts are almost negligible (< 1 nm). By contrast, the specific binding of 100 ng/ml MG on the nanosensor resulted in a significant shift of ca. 25 nm. Clearly, this positive signal confirmed that the present MG bioprobe has a sufficient selectivity required for accurate quantification.

Simultaneous cTnI and myoglobin detection

By immobilizing different antibody to respective nanorods demonstrating distinct LSPR band maxima, multiplex biosensing can be designed where multiple target molecules can be detected simultaneously. We found that the plasmon peak needs to be at least 150 nm separation to avoid indistinguishable longitudinal SPR bands after mixing. In this work, the myoglobin sensor was developed using the nanorods with resonance peak band at 640 nm, while the cTnI sensor was based on GNRs of 830 nm absorption. This is because the clinical

sensing range for cTnI protein is 0.1 – 10 ng/ml, which is much lower than the myoglobin (up to 300 ng/ml). Numerous studies have shown that the bulk refractive index sensitivity of the plasmon band is linearly correlated with the wavelength of the LSPR peak so that nanoparticles with a plasmon band at higher wavelengths are more sensitive to their local environment than those at shorter wavelengths. (Miller and Lazarides, 2005; Nusz et al., 2009) In view of these findings, anti-cTnI molecules were immobilized on the larger sized GNRs (i.e. 830 nm band maxima), while anti-MG was functionalized onto the smaller rods with 640 nm band. As shown in Fig. 3, the absorption spectrum of the two sized GNR mixtures showed three peaks. The first peak is the transverse absorption around 520 nm exhibited by both sized nanorods. The longitudinal SPR bands are combination of the varying sizes. A clear separation between the two distinct bands at 640 and 830 nm was observed, providing an ideal multiplexed biosensing platform.

To demonstrate simultaneous detection, a series of MG and cTnI samples at various concentrations were probed by the functional GNR sensor mixture. A spectral shift at 640 nm would indicate a specific binding of myoglobin and a red shift at 830 nm would be caused by cTnI binding. Fig. 4 shows the absorption spectra for different detection scenarios. The plasmon peak near 520 nm is not sensitive to perturbation in the surrounding refractive index, thus the spectral shift at this wavelength is negligible. When the sample contained only MG at 100 ng/ml (panel A), a 25-nm red shift at 640 nm was recorded due to target binding. As a comparison, no shift in the 840 nm plasmon band was observed due to the lack of cTnI molecules. Similarly, if only cTnI was spiked in the sample, a 5-nm spectral shift near 830 nm was demonstrated caused by specific binding of cTnI (panel B). The shift magnitude is much smaller than myoglobin because of an extremely low cTnI concentration at 5 ng/ml. When both analytes were present, the target binding on respective GNR probes resulted in pronounced shifts at both LSPR wavelengths simultaneously (panel C and D). The red shift is nicely correlated with the respective concentrations of MG and cTnI in the mixture. Similar to the single analyte detection, the spectral shift is a function of the target molecule concentration. As shown in Fig. 5, increase in the concentration caused larger shift in the respective GNR sensor, thereby providing a quantitative assessment of MG and cTnI simultaneously in the clinical range. The sensing performance was little affected by protein mixtures in the sample as compared to pure analyte detection. At higher concentrations, dips in the signal intensity in multiplex sensing were observed. This is normal in the diffusion-limited immunoassay where antibody is immobilized on solid substrates. (Tang et al., 2004) Nevertheless, the signal drop was not significant to adversely affect the sensing performance. Again, the results consistently indicate a superior specificity of the antibody moieties immobilized on the respective GNR sensors.

It was previously reported that end-to-end or side-by-side aggregation of nanorods could result in large shift of longitudinal SPR. (Jain et al., 2006a) For example, an aggregation of streptavidin with biotinylated nanorods caused a red shift of ~ 100 nm together with a significant band broadening. (Yu and Irudayaraj, 2007) This is because of the four active biotin-binding sites of the streptavidin molecules to crosslink nanoparticles as a cluster. In our study, the antibody (anti-MG or anti-cTnI) has only one active binding site to which the target analyte can interact due to steric hindrance. As such, the chances of red shift caused by GNR aggregation are greatly reduced. Moreover, the aggregation induced shift not only

in the longitudinal peak, but also in the transverse band. The morphology of the spectra was also distorted with pronounced peak broadening. As seen from Fig. 4, there was no transverse shift or significant broadening of both peaks after the MG and cTnI biosensing, thereby confirming the plasmon shifts observed were indeed due to specific binding events.

Finally, we would like to comment on the advantages of gold nanorods as the plasmonic transducer. First, in sensor applications, the changes in the plasmonic resonance wavelength of maximum absorption are monitored as a function of target analyte binding on the surface of nanoparticles. Thus, high sensitivity of the spectral response of the resonance absorption band to the changes in the refractive index of the surroundings is desired. Several studies have reported that for anisotropic nanoparticles, the shift in resonance wavelength is more sensitive and pronounced to the local refractive index than nanospheres. (Zeman, 2011; Yang, 1995; Malinsky, 2001; Sun and Xia, 2002) From the viewpoint of sensitivity, therefore, nanorod structure has been at the center of attention. While the wavelength shift of the plasmonic peak of spherical particles is only 1~2 nm which is too small a value for any realistic detection, gold nanorod bioprobes have demonstrated 10-50 nm red shift upon specific target binding. (Yu and Irudayaraj, 2007; Marinakos et al., 2007; Nusz et al., 2008; Tang et al., 2013; Casas et al., 2013) Second, we chose gold over silver because the greater reactivity of silver makes it less suitable for use in biological media, even though silver NPs exhibit higher plasmon sensitivity. (Lee and El Sayed, 2006) Third, gold nanorods can be conveniently synthesized with dominant resonance wavelength ranging from 600 to 1,100 nm simply by tuning their aspect ratio and size controlled by chemical synthesis (shown in Fig. 1). This wavelength range is attractive because the background absorption and scattering of endogenous chromophores from biological mixtures (e.g., serum and blood) is minimal. (Weissleder, 2001) This “biological window” is ideal for implantable optic biosensing and in vivo imaging development in future efforts. More importantly, the tunability of the plasmonic peak wavelengths provides a solid foundation to implement multiplexed biosensing in a simple yet effective way as demonstrated in this study. Lastly, gold nanorods can be efficiently assembled onto silanized glass substrates. (Wang and Tang, 2013) The GNR monolayer provides an excellent biosensing platform in a chip-based format, which can be easily utilized for miniaturization to deliver point of care medical diagnostics. Compared to the macroscale SPR sensors using a planar gold film and benchtop analytical instrument (e.g. Biacore), the nanorod based plasmonic sensor is superior in portability and flexibility of multiplexed biosensing.

Conclusions

We demonstrated the practical use of a multiplexed biosensor based on gold nanorods as the optical transduction of biological bindings for real protein detection. Clinically significant cardiac biomarkers (cTnI and myoglobin) were used as a model to simultaneously detect in various sample mixtures. To construct a multiplexed GNR bioprobe, nanorods were first synthesized with varying aspect ratios, exhibiting the longitudinal band maxima which is tunable from 600 to 1,100 nm. A mixture of GNRs with a separation of at least 150 nm in plasmonic wavelength allows multiplexed biosensing. Specifically, GNR bioprobes with 640 nm LSPR peak was used for myoglobin detection, while 830 nm for cTnI. Spectral shift at the respective wavelength indicates binding of target analyte with the specific antibody

immobilized on the nanosensor. The multiplexed GNR nanosensor is capable of simultaneously quantifying MG and cTnI concentrations in the clinical range with a high selectivity. This proof-of-concept study lays a solid foundation for further optimization of this promising technique for assay of physiological samples, which is the focus of current efforts. It is expected that this miniaturized nanosensor would greatly facilitate the point-of-care assay of multi-biomarkers within single sample for quick and reliable medical diagnostics. Moreover, the detection mechanism is universal for field-portable analysis of multiple targets in environmental protection, food safety and homeland security.

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Research Highlights

- Label free protein detection using gold nanorod (GNR) bioprobe is described.
- Tunability of the GNR longitudinal plasmon band enables multiplexed biosensing.
- Practical use for multiplexed cardiac biomarkers biosensing is demonstrated.

Figure 1.

A: Effect of the gold nanorod aspect ratio on peak wavelength of longitudinal surface plasmon resonance. B: absorption spectra of nanorods of different sizes. The longitudinal plasmon bands is tunable from 600 to 1,100 nm by adjusting the aspect ratio (AR).

Figure 2.

A: Standard calibration of longitudinal plasmon shifts as a function of increasing myoglobin concentration. At least three replications were measured to calculate the mean $\pm$ standard deviation for each concentration. B: High specificity of the MG (100 ng/ml) nanosensor when probed to various samples containing irrelevant molecules other than the target analyte.

Figure 3.

Absorption spectrum of nanoparticle mixture of two sized nanorods, showing longitudinal SPR bands at 640 and 830 nm, respectively. The shorter wavelength at 640 nm is dedicated for myoglobin (MG) assay, while the longer one for cardiac troponin I (cTnI) assay.

Figure 4.
Multiplexed detection of MG and cTnI at different combination.

Figure 5. Comparison of the multiplexed biosensing performance of myoglobin (panel A) or cTnI (panel B) sensor in single-analyte and multi-analyte samples in the targeted sensing range respectively.