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ORIGINAL PAPER

Enhanced Fluorescence Anisotropy Assay for Human Cardiac Troponin I and T Detection

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Abstract Human cardiac troponin I (hcTnI) and troponin T (hcTnT) are the biomarkers of choice for the diagnosis of cardiac diseases. In an effort to improve assay sensitivity, in this study we developed a novel approach to simultaneously detect hcTnI and hcTnT in homogenous solutions by monitoring enhanced-fluorescence-anisotropy changes. Specifically, our design was based on a competition assay by measuring anisotropy change of fluorophore-labeled peptides bound to primary monoclonal antibodies in the presence of nano-gold-modified secondary antibody in response to the presence of target proteins. Enhanced-fluorescence-anisotropy resulted from interaction between the primary antibody and the nano-gold-labeled secondary antibody, which significantly increased the size and decreased tumbling motion of the complex of peptide-antibodies. The measurements were performed to detect hcTnI and hcTnT either individually or simultaneously in a homogenous buffer solution and in the solutions containing human plasma. Our results showed that when fluorescence emission was monitored at a single wavelength selected by a monochromator the assay at all experimental conditions had excellent linear response to the target proteins within the concentration range of 0.5–40 nM. The detection limit is 0.5 nM for both hcTnI and hcTnT in the presence of human plasma. However, when fluorescence emission was monitored using a cutoff filter, the linear response of the assay to the target proteins is within 15–500 pM. The detection limit is 15 pM which is close to the recommended 99th percentile cutoff point for concentrations of hcTnI and hcTnT tests to discriminate healthy and diseased conditions. Homogenous nature, rapid response time, and easy implementation of our assay design make it a useful tool for disease biomarker and protein sensing.

Keywords Fluorescence anisotropy/polarization · Cardiac biomarker · Cardiac troponin · Peptide-base immunoassay · Competition assay

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Abbreviations

hcTnI	human cardiac troponin I
hcTnT	human cardiac troponin T
AMI	acute myocardial infarction

Introduction

Heart disease, including acute myocardial infarction (AMI), ranks at the top on the list of the most serious health problems in the United States [1]. Early diagnosis of AMI

is essential for successful medical treatment of the disease. Human cardiac troponin I (hcTnI) and troponin T (hcTnT) have been considered gold-standard cardiac biomarkers for heart disease diagnosis [2]. The rationale of using cardiac biomarkers for definitive diagnosis of heart disease is that upon cardiac muscle injury, the damaged myocytes disperse their cellular contents into the blood. The distinguishing characteristic of the cardiac biomarkers is that they are cardiac-specific proteins that permit discrimination of cardiac damage from damage to skeletal muscle or other organs. hcTnI and hcTnT are the subunits of cardiac troponin which play a central role in regulation of heart function. Since they are cardiac-specific proteins, the baseline concentration of hcTnI and hcTnT is undetectable in the blood of normal healthy individuals [3]. When AMI occurs, hcTnI and hcTnT are released into the bloodstream within the first few hours after onset of myocardial injury with peak concentrations typically occurring 12–24 h after onset [3]. The relative increase of hcTnI and hcTnT in the blood is higher than that observed for other cardiac biomarkers [4, 5]. To improve early detection of cardiac diseases, recent consensus recommendations have adopted a concentration of the troponin biomarkers above the 99th percentile of a healthy population to diagnose cardiac conditions [6]. Therefore, there is a need to develop new approaches to meet the new standard.

In an effort to detect cardiac biomarkers, various sensing approaches have been developed. Due to its high specificity and sensitivity, the immunoassay is employed in most of the sensing designs to sense the presence of the biomarkers [7, 8]. Typically these sensing designs use enzymatic-induced substrate color changes [8, 9], Förster resonance energy transfer (FRET) [7, 10], and surface plasmon resonance (SPR) [11, 12], as well as electrochemical and piezoelectric signals [4, 13] as signal read-out. Despite the extensive development of these sensors, each design has its own strengths and disadvantages. For example, in addition to their sensitivity and selectivity, the optical sensors based on SPR and ELISA involve laborious multi-step sample preparation processes, and electrochemical sensors require the immobilization of recognition molecules on sensing plates. Sensor based on the FRET technique requires both a fluorescent donor and acceptor labeling. Therefore, it is highly desirable to develop sensors with fewer preparation steps that still provide uncompromising sensitivity and reliability.

Peptide-based fluorescence anisotropy immunoassay has the characteristics of homogenous detection with simple fluorophore labeling and high sensitivity, which make it a promising approach for sensing protein-protein interactions. Fluorescence anisotropy assay is a versatile technique based on fluorescence anisotropy measurements, and it is now extensively used in various applications [14–16]. It is more robust than conventional intensity measurements due to its ratiometric nature, and to some extent it is not sensitive to fluctuations in concentration, light source, or sample thickness. Fluorescence anisotropy assays are carried out in homogeneous samples which do not require separation and do not require attachment to an immobilized phase.

In this report, we explored the feasibility of implementing peptide-based fluorescence anisotropy immunoassay in the detection of human hcTnI and hcTnT from serum plasma. Specifically, we aim to monitor binding events between primary monoclonal antibodies from mouse and synthesized peptides in homogenous solution in the presence of nano-gold-particle-labeled secondary anti-mouse antibodies by measuring fluorescence anisotropy changes of fluorescence probes that are attached to the synthesized peptides. The presence of full length target proteins in a sample solution can then be detected using analytic competition assay. Since the full length proteins have higher binding affinity to antibodies than peptides do, the presence of target proteins will result in fluorescence anisotropy changes of the fluorophore-labeled peptides. Because interaction between the primary antibody and the nano-gold-labeled secondary antibody significantly increases size and decreases tumbling motion of the peptide-antibody complexes, the measured fluorescence anisotropy change induced by the presence of the target protein is expected to be significantly increased. Our results showed that the peptide-based fluorescence anisotropy competition assay linearly responded to the presence of troponin biomarkers within picomolar concentration range and provided a promising sensing design for protein detection.

Experimental Methods

Materials and Reagents OneShot® BL21 Star™ (DE3) chemically competent *E. coli* cells were purchased from Invitrogen. 5-Carboxyfluorescein (5-FAM) was purchased from Sigma-Aldrich (St. Louis, MO). Texas Red®-X, succinimidyl ester (mixed isomers) was purchased from Invitrogen Corporation (Carlsbad, CA). Peptides were synthesized by the Molecular Biology Core of Washington State University. Mouse monoclonal cardiac troponin I antibody (ab19615) was obtained from Abcam (Cambridge, MA). Mouse monoclonal cardiac troponin T antibody (NB120-10213) was purchased from Novus Biologicals (Littleton, CO). Anti-mouse IgG secondary antibody labeled with 10 nm gold particle was either purchased from Sigma-Aldrich or prepared in our lab. Briefly, the colloidal gold solution (1% w/v) (BB Intentional) was adjusted to pH 8.5 with 0.1 M Na_2CO_3. Then, 1.5 mg rabbit polyclonal secondary antibody to mouse IgG (4.3 mg/ml in phosphate buffer, 10 mM, pH 7.2) purchased from Abcom

was added to the 50 ml pH-adjusted colloidal gold solution drop by drop. The mixture was smoothly mixed for 60 min. Then, 2 ml of 10% BSA solution was added in order to block the residual surface of the nanocolloidal gold particles. The obtained solution was stirred for 15 min and then was centrifuged at 15,000 rpm for 45 min at 4 °C. After centrifugation, the pellet was suspended in 50 ml dilution phosphate buffer (10 mM buffer pH 7.2 containing 1% w/v BSA). The resultant solution was then centrifuged for the second time under the same condition. The pellet was resuspended in 5 ml dilution phosphate buffer (10 mM sodium phosphate pH 7.2 containing 1% w/v BSA and 0.1% sodium azide) and the optical density was adjusted to 8.0 at 520 nm with the dilution buffer. This anti-mouse IgG labeled with colloidal nano-gold was stored at 4 °C.

Wild Type Human Cardiac Troponin I and Troponin T Purification

pET3d clone of hcTnI or hcTnT was transformed into 20 µl OneShot® BL21 Star™ (DE3) Chemically Competent *E. coli* cells and grown in 15 ml TB medium with 50 µg/ml carbenicillin at 37 °C for 4 h. The preculture was inoculated into 2 L TB medium with 50 µg/ml carbenicillin and the cells were grown at 37 °C for 18 h. The cells were harvested and sonicated in a buffer containing Triton X-100, 0.01% NaN_3, 1 mM PMSF and 1 mM Benzamidine. After centrifugation, the supernatant was subject to fractionation of 30%–60% $(NH_4)_2SO_4$. For hcTnI purification, the pellet was suspended in a 50 ml CM buffer containing 6 M Urea, 50 mM citrate, pH 6.4, 1 mM EDTA, 1 mM DTT, and 0.2 M KCl. The sample was dialyzed against 1 L CM buffer overnight at 4 °C to remove residual $(NH_4)_2SO_4$. The sample was loaded to equilibrated carboxy methyl (CM) sepharose column. The protein was eluted with NaCl gradient from 0 to 0.3 M. For hcTnT purification, the pellet was resuspended in 50 ml of DEAE buffer containing 6 M Urea, 50 mM tris, pH 7.8, 1 mM EDTA, 1 mM DTT, and 0.2 M KCl. It was then dialyzed against 1 L DEAE buffer overnight at 4 °C to remove residual ammonium sulfate. Dialyzed supernatant was loaded into diethylaminoethyl (DEAE) sepharose column, and the protein was eluted with NaCl concentration gradient from 0 to 0.5 M. The purity of the proteins was checked with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Specificity of the purified proteins binding to corresponding monoclonal antibodies was examined using western blotting techniques.

Peptide Synthesis and Labeling Two peptides were synthesized by the Molecular Biology Core facility in Washington State University. The first peptide is a 15 amino acid sequence from 40th to 54th amino acid of full length hcTnI sequence: 40-KISASRKLQLKTLLL-54 (peptide A), where the epitope region of corresponding hcTnI antibody (ab19615) is from 41st to 49th amino acid (the underlined sequence). The second peptide is a 19 amino acid sequence from 145th to 163rd amino acid of full length hcTnT sequence: 145-EKERQNRLAEERARREEEE-163 (peptide B), where the epitope region of corresponding hcTnT antibody (NB120-10213) is from 146th to 160th amino acid (the underlined sequence). The synthesized peptides A and B were labeled with 5-FAM and Texas Red, respectively, at the N-termini, followed by separating the labeled peptides from the unlabeled peptides using a high performance liquid chromatography. FAM and Texas Red were chosen because of their well-separated emission spectra (Fig. 1), which make them good candidates for multiple detections in homogenous solutions. Both synthesized peptides and labeled products were verified using mass spectrometry (data not shown). The function of the labeled peptides binding to the corresponding monoclonal antibodies was verified by measuring changes in fluorescence anisotropy of the fluorophores-labeled peptides in the absence and presence of the corresponding antibodies.

Fluorescence Measurements The steady-state fluorescence emission measurements were carried out at 10±0.1 C on an ISS PCI photon-counting spectrofluorometer [17]. The steady-state fluorescence anisotropy was measured with a L-format instrumental setup. The sample containing FAM-modified hcTnI peptide A in a phosphate buffer solution was excited with a vertically-polarized light of 488 nm, and the emission was monitored at 530 nm. The sample containing Texas-Red-modified hcTnT peptide B was

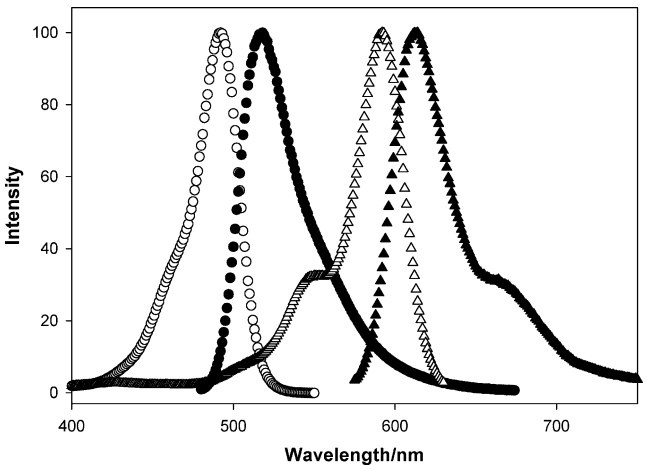


Fig. 1 Absorption and emission spectra of fluorophore-labeled peptides. Absorption and emission spectra are shown in open-sign curves and solid-sign curves, respectively, The circle-sign curves are measured from FAM-labeled hcTnI peptide A and the triangle-sign curves are obtained from Texas Red-labeled hcTnT peptide B in PBS solutions

excited with a vertically-polarized light of 580 nm, and the emission was monitored at 630 nm. Fluorescence intensities of vertically-polarized emission ($I_{||}$) and horizontally-polarized emission ($I_{\perp}$) were measured from emission channel by alternatively orientating polarizer at vertical and parallel positions. The total fluorescence intensity I can also be determined from the same data with the equation $I = I_{||} + 2I_{\perp}$. The anisotropy (r) was calculated using Eq. 1:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (1)$$

Results and Discussion

Characterization of Protein-Antibody Interactions

After ammonia sulfate fractionation and chromatographic purifications, the identities and purities of the obtained hcTnI and hcTnT were checked with SDS-page gels (Fig. 2). Both proteins showed adequate purity for fluorescence anisotropy sensing measurements. To characterize protein-antibody interactions, we performed western blotting to verify specificities of monoclonal antibody ab19615 against hcTnI and antibody NB120-10213 against hcTnT (Fig. 2). The two antibodies ab19615 and NB120-10213 used in this experiment are specific for hcTnI and hcTnT, respectively. To verify the specificity, we also performed cross-reaction of the hcTnI antibody with rat cTnI (RcTnI), and the cross-reaction of the hcTnT antibody with rat cTnT (RcTnT) and rat skeletal TnT (RsTnT). The results shown in Fig. 2 suggested the antibodies ab19615 and NB120-10213 are highly specific to hcTnI and hcTnT, respectively. These results lay the foundation for us to use these two antibodies for hcTnI and hcTnT detection.

Characterization of the Synthesized Peptide-Antibody Interactions

The synthesized peptides A and B contain epitope region of hcTnI and hcTnT for antibodies ab19615 and NB120-10213, respectively. Therefore, they should specifically interact with their corresponding monoclonal antibodies. To verify the specific interactions, we performed fluorescence anisotropy measurements to monitor the binding of the antibodies to their target peptides. These measurements are based on the fact that fluorescence anisotropy of the fluorophore attached to the peptide will be significantly increased upon binding to the antibody because of significant increases in the size of the molecular complex and reductions in the mobility of the peptide. Steady-state fluorescence anisotropy of FAM-labeled hcTnI peptide A at 40 nM concentration was measured with a fixed excitation at 480 nm and emission at 530 nm in a PBS buffer (pH 7.4). The anisotropy was 78 mP in the absence of the corresponding antibody, suggesting a rapid tumbling of the peptide in solution. When the antibody ab19615 was introduced into the sample, the anisotropy started to increase and to reach a maximum (170 mP) at a molar ratio of peptide:antibody of 2:1. Similar fluorescence anisotropy measurements with excitation 592 nm and emission at 621 nm showed that anisotropies of Texas-Red-labeled hcTnT peptide B were 99 mP without presence of the corresponding antibody NB120-10213 and 151 mP with presence of the antibody (Table 1). The anisotropy of the peptide B showed a higher value (99 mP) than the

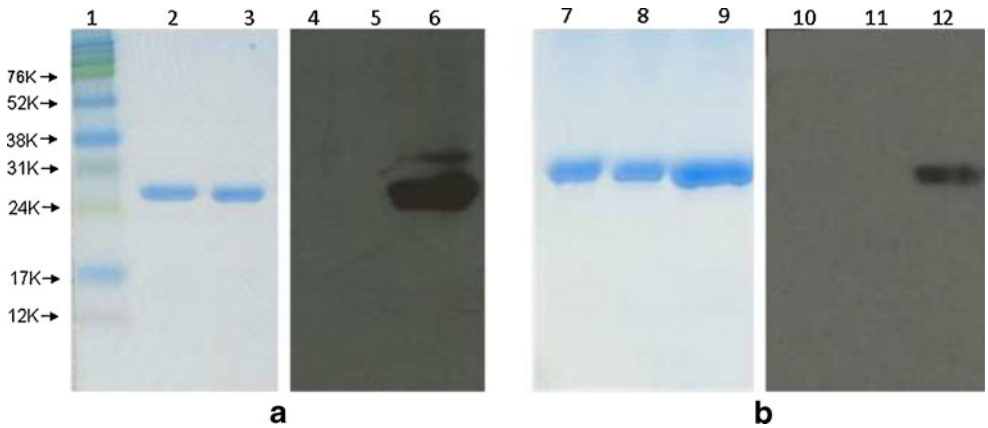


Fig. 2 Electrophoresis analysis of hcTnI and hcTnT. **a** SDS-PAGE gels (12% resolving and 4% stacking) is on the left and Western blotting image is on the right. Lanes 1: Standard protein rainbow markers; 2: rat cTnI; 3: purified hcTnI; 4–6: Western blotting images of rainbow marker (lane 1), rat cTnI (lane 2) and hcTnI (lane 3), respectively, in the presence of antibody ab19615. **b** SDS-PAGE gels (12% resolving and 4% stacking) are on the left and Western blotting image is on the right. Lanes 7: rat cTnT; 8: rat skeletal TnT; 9: purified hcTnT; 10–12: Western blotting images of rat cTnT(lane 7), rat skeletal TnT (lane 8); and purified hcTnT (lane 9), respectively, in the presence of antibody NB120-10213 which is specific for hcTnT. The gel images on both panels are referenced to the same rainbow protein markers on lane 1 of panel a

Table 1 Changes in fluorescence anisotropy (FA) of fluorophore-labeled peptides upon formation of antibody-peptide complexes

Sample	FA (peptide only)	FA (antibody-peptide complex)	FA changes (%)
Peptide A only without secondary antibody[a]	76 mP[c]	170 mP	123.7
Peptide B only without secondary antibody[a]	99 mP[c]	151 mP	53.2
Peptide A only in the presence of secondary antibody[b]	81 mP[d]	269 mP	232.1
Peptide B only in the presence of secondary antibody[b]	103 mP[d]	247 mP	139.8
Peptide A in the presence of peptide B with secondary antibody[b]	85 mP[d]	276 mP	224.7
Peptide B in the presence of peptide A with secondary antibody[b]	108 mP[d]	268 mP	148.2
Peptide A in the presence of peptide B & human plasma & secondary antibody[b]	109 mP[d]	288 mP	164.2
Peptide B in the presence of peptide A & human plasma & secondary antibody[b]	118 mP[d]	277 mP	134.8

[a] Concentrations of fluorophore-labeled peptides were 40 nM

[b] Concentrations of fluorophore-labeled peptides were 30 nM

[c] Measurements were performed in the absence of either primary or secondary antibodies

[d] Measurements were performed in the presence of nano-gold-labeled anti mouse IgG secondary antibodies

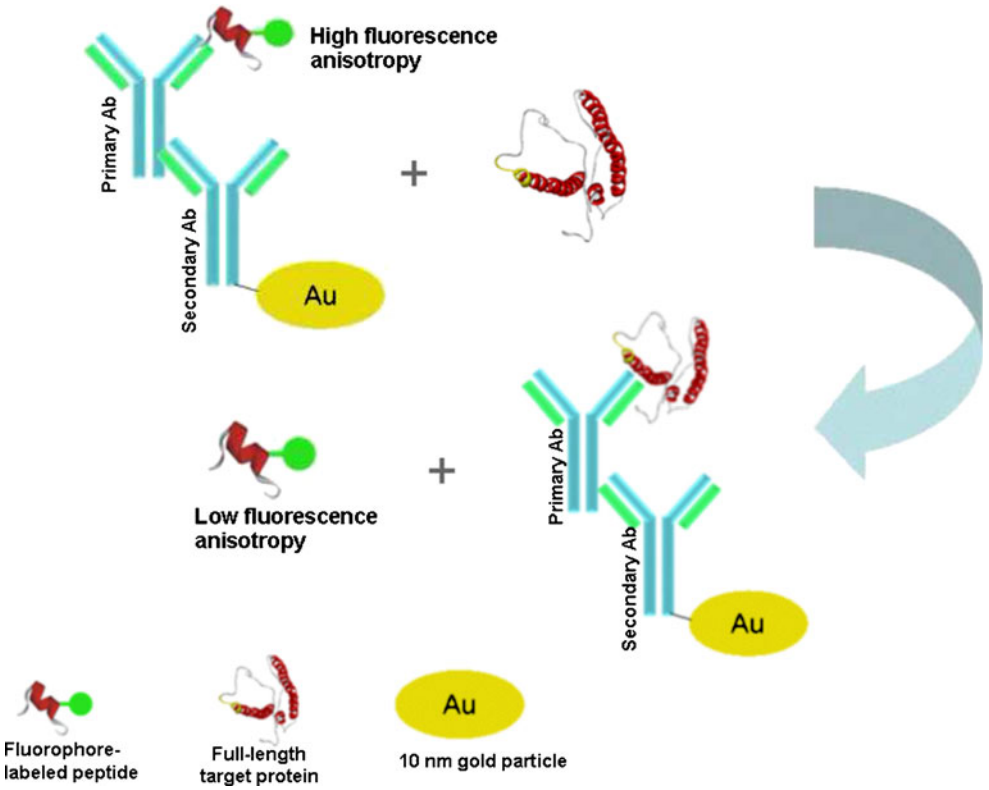
peptide A (76 mP); this is because peptide B has a higher molecular weight (3.74 kDa) than peptide A (2.46 kDa). The significant increases in the anisotropies in the presence of antibodies reflect larger size and slow rotation of the molecules, which in turn led to increases in anisotropy of the molecule according to the Perrin equation [18]. This suggests that the synthesized peptides are able to effectively bind to the corresponding antibodies.

Fluorescence Anisotropy Enhancements by the Presence of Nano-Gold-Labeled Secondary Antibody

To further improve signal/noise ratio of fluorescence anisotropy measurements, an excess amount of nano-gold-labeled secondary anti mouse antibody was added to the mixed samples containing either the synthesized peptides A and hcTnI primary monoclonal antibody ab19615 (from mouse) or the synthesized peptide B and hcTnT primary monoclonal antibody NB120-10213 (from mouse). The interaction between the primary antibody and nano-gold labeled secondary antibody significantly increased masses and reduced tumbling motion of the complexes, leading to enhancements of fluorescence anisotropies of the fluorophore-labeled peptides. Steady-state fluorescence anisotropy measurements (Table 1) showed that fluorescence anisotropies of FAM-labeled hcTnI peptide A and Texas-Red-labeled hcTnT peptide B at 30 nM concentration in a PBS buffer (pH 7.4) was 81 mP and 103 mP, respectively, in the presence of nano-gold-labeled secondary antibody, but without the presence of primary antibody. The presence of nano-gold-labeled secondary antibody alone had no effect on fluorescence anisotropies of the fluorophore-labeled peptides in solutions. Additions of primary antibodies to the samples containing fluorophore-labeled peptides and nano-gold-labeled secondary antibodies increased fluorescence anisotropies of FAM-labeled hcTnI peptide A and Texas-Red-labeled hcTnT peptide B to 269 mP and 247 mP, respectively (Table 1). The significant increases in the anisotropies reflect larger sizes and slow rotations of the complexes of peptide-primary-antibody-secondary-antibody (Scheme 1). The large changes in fluorescence anisotropies provide a foundation for our design to utilize a competition assay to detect both hcTnI and hcTnT in homogenous samples.

Competition Assay to Sense hcTnI and hcTnT in Homogenous Solutions

We use a fluorescence anisotropy change resulting from interruption of the peptide-antibody interaction by the target protein as a read-out signal to sense the presence of hcTnI and hcTnT in homogenous solutions. The sensing design is schematically presented in Scheme 1. Our rationale for the sensing design is that the full-length target hcTnI and hcTnT have higher binding affinities to the corresponding antibodies, therefore interrupting the peptide-antibody interactions. Displacing the peptide from the binding sites of the primary antibody leads to a significant decrease in the fluorescence anisotropy of the fluorophore attached to the peptide. To test our hcTnI assay, we incrementally increased hcTnI concentration from 0.1 nM to 60 nM in an assay solution containing 30 nM FAM-labeled peptide A, 18 nM primary antibody and 20 nM nano-gold-labeled secondary antibody (insert of Fig. 3). Our results showed that the fluorescence anisotropy (269 mP) of the peptide did not change when the concentration of hcTnI was below



Scheme 1 Schematic presentation of the design for target protein detection using enhanced fluorescence anisotropy measurements

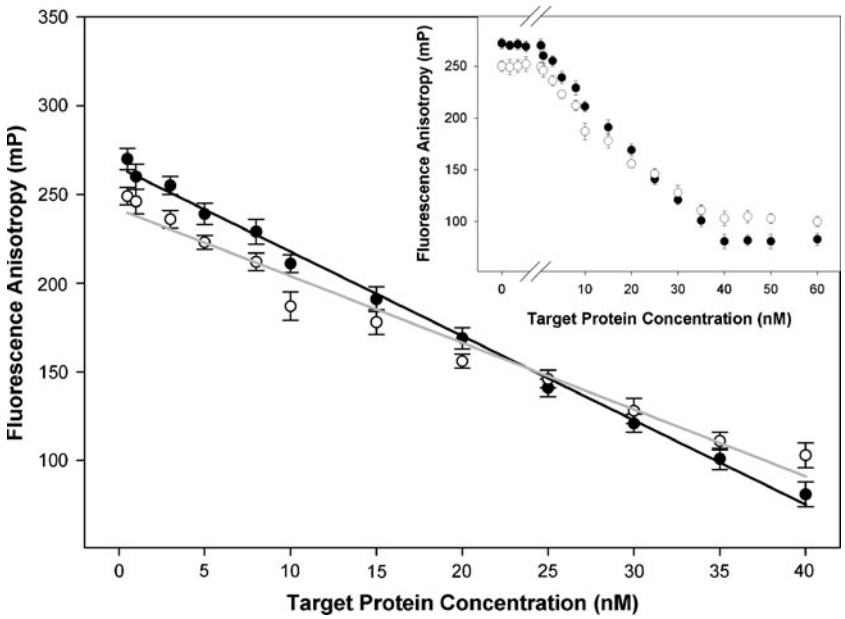


Fig. 3 Individual competition assay of hcTnI and hcTnT in BPS buffers. Black dots are experimental data for hcTnI detection in the presence of 30 nM FAM-labeled peptide A and 18 nM primary monoclonal antibody ab19615 (from mouse) plus 20 nM nano-gold-labeled secondary anti-mouse antibody. Fluorescence emission was monitored at a single wavelength selected using a monochromator. The black line is fitting data for the linear region of hcTnI titration, which gives the slope of −4.75 with R^2=0.996. Open circles are experimental data for hcTnT detection in the presence of 30 nM Texas-Red-labeled peptide B and 18 nM primary monoclonal antibody NB120-10213 (from mouse) plus 20 nM nano-gold-labeled secondary anti-mouse antibody. The gray line is fitting data for the linear region of hcTnT titration, which gives the slope of −3.76 with R^2=974. Insert shows experimental data for either hcTnI or hcTnT titrations within concentration range from 0.1 nM to 60 nM. The vertical bars represent standard deviations with three sets of measurements

0.5 nM and linearly decreased to 81 mP when concentration of hcTnI was between 0.5 nM and 40 nM with a decrease slope of −4.75 (Fig. 3). Further increase in concentration of hcTnI had no effect on the fluorescence anisotropy because all binding sites of antibodies had been saturated. Also shown in Fig. 3 are fluorescence anisotropy changes of Texas-Red-labeled hcTnT peptide B (30 nM) in the presence of primary antibody (18 nM) and nano-gold-labeled secondary antibody (20 nM) with increasing concentration of hcTnT from 0.1 nM to 60 nM. The fluorescence anisotropy was not changed when the concentrations of hcTnT were below 0.5 nM and above 40 nM. The anisotropy was linearly decreased with a slope of −3.76 when the concentration of hcTnT increased from 0.5 nM to 40 nM.

The results demonstrated that using the competition assay based on enhanced fluorescence anisotropy measurements with fixed emission wavelength we can accurately detect nanomolar concentrations of either hcTnI or hcTnT in homogenous solutions. However, these results were obtained from separate individual measurements of either hcTnI or hcTnT. The objective of this study is to develop a general assay for dual detection of both hcTnI and hcTnT in the same sample. To examine whether the two (hcTnI and hcTnT) binding events interfere with each other in detection of the corresponding target proteins, we performed the anisotropy measurements for each detection in the presence of the other. Our results showed that in the presence of the other binding event, the anisotropy of peptide A increased from 85 mP to 276 mP upon binding to the corresponding antibody ab19615, and the anisotropy of peptide B increased from 108 mP to 268 mP upon binding to the corresponding antibody NB120-10213 (Table 1). Magnitudes of the anisotropy changes are similar to the changes observed in each individual measurement. Competition assay measurements showed good linearity and sensitivity for both hcTnI and hcTnT detection (Data not shown). Linear ranges for both protein detections are similar to the results obtained from each individual protein detection with similar sensitivity, i.e., 0.5 nM–40 nM for both hcTnI and hcTnT assays. These results demonstrate that this competition assay can be of use for dual detection of hcTnI and hcTnT in a homogenous sample preparation.

Detection of hcTnI and hcTnT in Homogenous Human Plasma Solutions

One of our objectives in this study is to explore whether our fluorescence anisotropy-based competition assay can be used for in vitro hcTnI and hcTnT simultaneous detection. To achieve this objective, the interactions between the fluorophore-labeled peptides and the corresponding primary antibodies in the presence of nano-gold-labeled secondary antibodies were monitored by measuring fluorescence anisotropy changes of the peptide-fluorophores in a homogenous solution containing 10% human plasma. Our results showed that the anisotropy measurements were significantly interfered by high scattering background from the plasma (data not shown), which contains numerous abundant proteins. Typically, the plasma contains about ten high-abundance proteins which together represent about 97% of the total plasma proteins. The most abundant proteins are albumin and the immunoglobulin heavy and light chains (IgG), representing together about 80% of the plasma proteins. Therefore, depletion of these two high abundance proteins may significantly decrease scattering background from solution medium.

To improve the signal/noise ratio of the fluorescence anisotropy measurements, the human plasma was passed through a HiTrap albumin & IgG depletion column (GE Healthcare) following manufacture protocol. The procedure removed about 95% of albumin and IgG proteins from the plasma. We also passed a sample containing hcTnI and hcTnT through the column to test whether the HiTrap column removes any hcTnI and hcTnT from the sample. Total concentration analysis (data not shown) suggests no binding of hcTni and hcTnT to the column. The binding of the fluorophore-labeled peptides to the corresponding antibodies in a homogenous solution containing 10% depleted plasma were studied. Without the presence of the primary antibodies, fluorescence anisotropy of the fluorophore-labeled peptide A and peptide B were 109 mP and 114 mP, respectively. The elevated anisotropies compared to the anisotropy obtained from non-plasma samples reflect the increased viscosity of the sample solution due to the presence of 10% of the deleted plasma, therefore slowing down tumbling time of the peptides in the solution. Upon binding to the primary antibodies, the fluorescence anisotropies of peptides A and B significantly increased to 288 mP and 277 mP, respectively, in the presence of nano-gold-labeled secondary antibodies (Table 1). Similar to the results shown in Fig. 3, competition assay measurements of the target hcTnI and hcTnT with the samples containing the depleted plasma showed good linearity (R^2=0.99 for hcTnI assay, and R^2=0.98 for hcTnT assay) within 0.5 nM to 40 nM concentration range, sensitivity (slope=−4.70 for hcTnI and slope=−4.03 for hcTnT), and reproducibility (Fig. 4).

Typically, concentrations of hcTnI and hcTnT circulating in blood can be undetectably low (0.01 ng/mL) when the heart is in healthy condition [19]. At a diseased state, four to six hours after cell death, both hcTnI and hcTnT released to circulating blood can be significantly increased and reached maximum within twelve-to-twenty-four hours. For

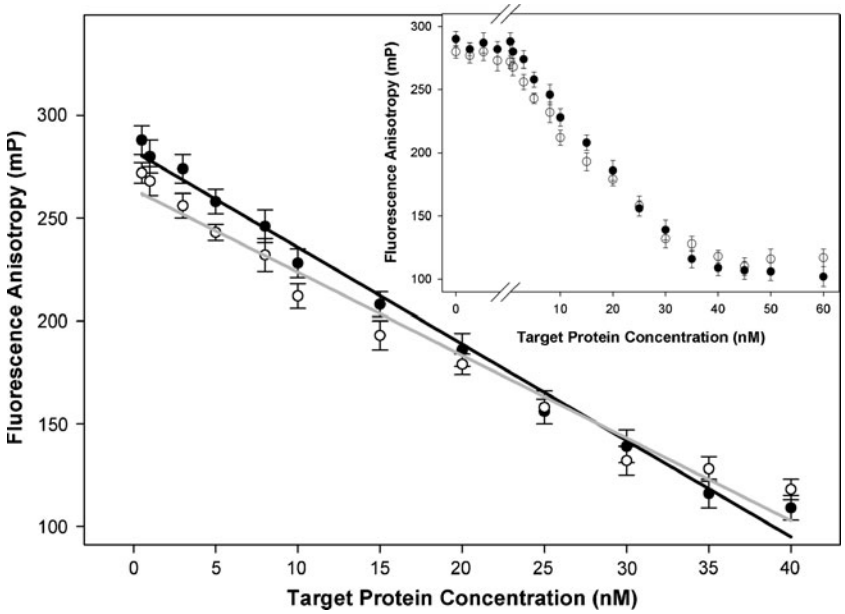


Fig. 4 Competition assay of mixed hcTnI and hcTnT in 10% depleted human plasma solution plus 40 nM nano-gold-labeled secondary anti-mouse antibody. Fluorescence emission was monitored at a single wavelength selected using a monochromator. Black dots are experimental data for hcTnI detection in the presence of 30 nM FAM-labeled peptide A and 18 nM primary monoclonal antibody ab19615 (from mouse). The black line is fitting data for the linear region of hcTnI titration, which gives the slope of −4.70 with R^2=0.992. Open circles are experimental data for hcTnT detection in the presence of 30 nM Texas-Red-labeled peptide B and 18 nM primary monoclonal antibody NB120-10213 (from mouse). The gray line is fitting data for the linear region of hcTnT titration, which gives the slope of −4.03 with R^2=0.975. Insert shows experimental data for either hcTnI or hcTnT titrations within concentration range from 0.1 nM to 60 nM. The vertical bars represent standard deviations with three sets of measurements

example, in response to an acute myocardial infarction, the concentration of hcTnI increases as much as 158 times in blood [20]. As the gold standard for diagnosis of cardiac diseases, the 99th percentile cutoff point for concentrations of hcTnI and hcTnT tests to discriminate healthy and diseased conditions are recommended as 0.01–0.08 ng/mL [6]. The stringent cutoff points of hcTnI and hcTnT concentration tests can significantly improve earlier detection and lower cardiac risk of patients. However, this also posts a challenge to assay sensitivities and detection limits of hcTnI and hcTnT detections. Even if 0.08 ng/mL is used as the cutoff point for concentrations of hcTnI and hcTnT tests, it will require the detection limit of 3.2 pM concentration of troponin biomarker in a blood sample, which is too low for many standard assays currently used. Our assay with 0.5 nM detection limit is not sensitive enough to meet the 99th percentile cutoff point requirement for concentrations of hcTnI and hcTnT tests.

The relatively high detection limit of our design is obtained from measurements in which fluorescence intensity was monitored at a monochromatically-selected single wavelength. To improve the sensitivity of our assay, we removed monochromator from the left detection channel and emission of FAM-labeled peptide A and Texas-Red-labeled peptide B was monitored using a 520 nm cutoff filter and a 600 nm cutoff filter, respectively. The new instrumental setting tremendously increase signal/noise ratio of fluorescence measurements, which enable us to lower the concentration of fluorophore-labeled peptides from 30 nM to 0.2 nM. To test our hcTnI assay with the new instrumental setting, we incrementally increased hcTnI concentration from 5 pM to 700 pM in a sample containing 0.2 nM FAM-labeled peptide A, 0.12 nM antibody and 0.5 nM nano-gold-labeled secondary antibody in the presence of 10% depleted plasma. Our results showed that the fluorescence anisotropy (280 mP) of the peptide did not change when the concentration of hcTnI was below 15 pM and linearly decreased to 77 mP when the concentration of hcTnI was between 15 pM and 500 pM with a decreased slope of −0.439 (Fig. 5). Further increase in concentration of hcTnI had no effect on the fluorescence anisotropy. Also shown in Fig. 5 are fluorescence anisotropy changes of Texas-Red-labeled hcTnT peptide B (0.2 nM) in the presence of antibody (0.12 nM), nano-gold-labeled secondary antibody (0.5 nM), and 10% depleted plasma with increasing concentration of hcTnT from 5 pM to 700 pM. The fluorescence anisotropy was not changed when the concentrations of hcTnT were below 15 pm and above 500 pM. The anisotropy was linearly decreasing with a slope of −0.327 when the concentration of hcTnT increased from 15 pM to 500 pM.

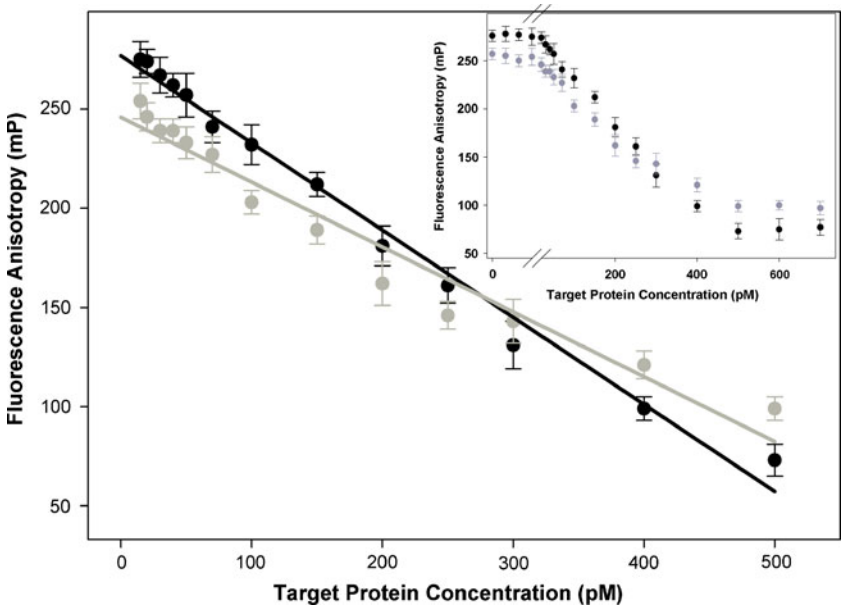


Fig. 5 Competition assay of mixed hcTnI and hcTnT with an improved instrumental setting in which fluorescence emission was selected using a cutoff filter rather than a monchromator used in measurements described in Figs. 3 and 4. The assay was performed in the presence of 10% depleted human plasma and 1 nM nano-gold-labeled secondary anti-mouse antibody. Black dots are experimental data for hcTnI detection in the presence of 0.20 nM FAM-labeled peptide A and 0.12 nM primary monoclonal antibody ab19615 (from mouse). The black line is fitting data for the linear region of hcTnI titration, which gives the slope of −0.439 with R^2=0.982. Open circles are experimental data for hcTnT detection in the presence of 0.20 nM Texas-Red-labeled peptide B and 0.12 nM primary monoclonal antibody NB120-10213 (from mouse). The gray line is fitting data for the linear region of hcTnT titration, which gives the slope of −0.327 with R^2=0.979. Insert shows experimental data for either hcTnI or hcTnT titrations within concentration range from 5 pM to 700 pM. The vertical bars represent standard deviations with three sets of measurements

In summary, an enhanced change of fluorescence anisotropy was implemented to develop this novel dual-sensing scheme for simultaneous detection of two cardiac markers, hcTnI and hcTnT. The enhanced fluorescence anisotropy was achieved by adding a nano-gold-modified secondary antibody to the assay samples containing complexes of fluorophore-labeled peptides and the corresponding primary antibody. Binding of the secondary antibody to the primary antibody significantly increase the size of the complexes, leading to higher fluorescence anisotropy of the complexes. All tests performed in either simple single-peptide or complex conditions mimicking in vitro blood sample demonstrated good linearity between anisotropy changes and sub-nanomolar range of both hcTnI and hcTnT in competition assays. Particularly, combining the enhanced fluorescence anisotropy and improved instrument setting (using cutoff filter instead of monochrmotor) our assay is able to detect both hcTnT and hcTnT as low as concentration of 15 pM, which is close to the recommended 99th percentile cutoff point for concentrations of hcTnI and hcTnT tests to discriminate healthy and diseased conditions. It is believed that further improvement in detection limits of hcTnI and hcTnT can be made with implementing a high power light source, such as a laser, into our design. Because of the ratiometric nature of homogenous assay and easy implementation, our enhanced fluorescence anisotropy assay design provides a novel format with good resolution for rapid and reliable multiple-cardiac biomarker detection in homogenous plasma samples. It can also be implemented as a general approach for multiple-protein detection in homogenous sample solutions.

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