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Metal-enhanced fluorescent biomarkers for cardiac risk assessment

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Abstract

In this presentation we describe a novel methodology for ultra-sensitive fluorescence immunoassays based on a new class of fluorescent biomarkers, which are strongly enhanced by nano-size metallic particles. Specifically, we discuss development of the immunoassay on the surfaces coated with metallic particles for high sensitivity detection of cardiac markers. This technology will allow detection of the biomarkers in serum and blood without separation and amplification steps. We present an experimental platform that uses front-face excitation in total internal reflection mode for efficient rejection of background fluorescence.

Keywords

fluorescence immunoassay; enhanced fluorescence; nano-size metallic particles; silver islands; cardiac markers

1. INTRODUCTION

Cardiovascular diseases are the leading cause of mortality in developed countries. In the United States alone, more than 500,000 people die each year from sudden cardiac arrest or from consequences of chronic heart complications. Reliable methods of detecting functional/physiological cardiac disorder in an early development stage would have a fundamental impact on today's preventative medicine. The use of various cardiac markers in clinical practice is actively debated 1–2. Most authors recommend not to rely on a single marker, but to use a combination of several markers (such as Myoglobin, CK-MB, Troponin I, and Troponin T), and repeat the serum marker testing during 9–10 hours after the disease symptom 3–4. Fluorescence-based immunoassays are extensively used in medical diagnostics 5–8. In general, the detectability of a fluorophore is determined by two factors, the photostability of the fluorophore and the background emission of the sample. Our approach to the fluoroimmunoassay increases the fluorescence intensity (relative to the background signal) and increases the number of detected photons per fluorophore. This approach is based on our recent studies of the metal-enhanced fluorescence showing that

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close proximity of the fluorophore to the metallic silver particles increases the brightness and photostability of the fluorophore 9–10. Here we present the application of this technology to the immunoassay for a model antigen and for Myoglobin.

2. METHODOLOGY

2.1. Materials

Rabbit Immunoglobulin G (IgG) and goat IgG (95% pure) were from “Sigma”. Tetramethylrhodamine-anti-Rabbit IgG (produced in goat) was from “Sigma” and “Molecular Probes”. Rhodamine Red-X- anti-Rabbit IgG conjugate, Alexa Fluor-647-antiRabbit IgG conjugate, Alexa Fluor-680-antiRabbit IgG conjugate and Alexa Fluor-750-antiRabbit IgG conjugate were from “Molecular probes”. Myoglobin (recombinant) and monoclonal anti-Myoglobin antibodies (capture anti-Myoglobin antibodies clone 3 2mb-295, reporter anti-Myoglobin antibodies clone 9mb-183r) were from “Spectral Diagnostics”, Canada. Capture antibodies were labeled with Rhodamine Red-X and Alexa Fluor-647 using labeling kits from “Molecular Probes”; dye/protein ratio was determined spectrophotometrically according to the kit instructions. Buffer components and salts (such as bovine serum albumin, glucose, sucrose, AgNO₃) were from “Sigma-Aldrich”

2.2. Silver islands formation

Silver islands film surface (SIF) was formed similar to the procedure described elsewhere 9,10. In particular, glass slides (“VWR”, 3×1 inch, 1 mm thick) were cleaned by soaking in a 9:1 mixture (v/v) of H₂SO₄ (98%) and H₂O₂ (30%) for at least overnight, then rinsed with Milli-Q water and dried on air before use. Slides were then coated with poly-L-lysine: ca. 1.2 mL of the poly-L-lysine solution (freshly prepared solution: 8 mL water + 1.0 mL Na-phosphate buffer, 50 mM, pH 7.4 + 1.0 mL poly-L-lysine solution (“Sigma”, 0.1%)) was added to each slide and spin-coated. Spin-coating was performed using P-6708D spin-coater (“Specialty Coating Systems”, USA) at 2000 rpm for 3 sec and at 3000 rpm for 1000 sec (time ramps to reach 2000 rpm (from 0) and 3000 rpm (from 2000) were 3 sec each, and time ramp to slow down from 3000 rpm to 0 was 10 sec). Silver deposition was performed in a glass beaker as follows. At intensive stirring, 10 drops of NaOH solution (5 M) were added to AgNO₃ solution (250 mg) in Milli-Q water (35 mL); dark-brown precipitate was formed immediately. About 1 mL of 30% NH₄OH solution was added to dissolve the precipitate (at continuous intense stirring), and the clear solution was cooled with ice to 10–15 °C (about 5 min). A fresh glucose solution (360 mg D(+)-glucose in 10 mL Milli-Q water) was added to the mixture, and glass slides were immediately half-inserted (half-soaked) into the mixture. Soaking of slides was performed for pairs of slides, so only one surface of each slide was exposed to the reaction mixture. Stirring of the mixture was continued in ice bath for 1 min, then ice was removed and solution stirred at warming (at medium heating) until 30°C for about 2 min. Then the heating was turned off and the solution was continued to be intensively mixed for about 3–4 more min (the temperature increased to 35°C). After the color of the slides became greenish-brown and solution became opaque, the slides were removed from the beaker and washed with water 2 times with sonication for about 30 sec. After rinsing several times with water, slides were stored in water at room temperature

(several hours to months) until use. A typical absorbance spectrum of the SIF-coated glass slide is given on Figure 1.

2.3. Coating slides with IgG (model immunoassay)

Slides were non-covalently coated with rabbit IgG as a sample slide, or goat IgG as a control slide. First slides were dried on air and covered with the tape containing punched holes (regular size hole puncher) to form wells on the surface of the slides. Coating solution of IgG (10–30 $\mu\text{g}/\text{mL}$ of IgG dissolved in Na-phosphate buffer, 50 mM, pH 7.4) was added to each well (25 μL per well), and slides were incubated for 2–4 hours at room temperature in humid chamber. Slides then were rinsed with water, washing solution (0.05% Tween-20 in water), and water. Blocking was performed by adding blocking solution (1% BSA, 1% sucrose, 0.05% NaN_3 , 0.05% Tween-20 in 50 mM Tris-HCl buffer, pH 7.4; 35 μL per well) and incubation at room temperature for 2–4 hours (or overnight at $+4^\circ\text{C}$) in humid chamber. Slides were rinsed with water, washing solution (0.05% Tween-20 in water), and water, covered with Na-phosphate buffer (50 mM, pH 7.4) or blocking solution and stored at $+4^\circ\text{C}$ until use.

2.4. Coating slides with anti-Myoglobin Ab/Myoglobin antigen (Myoglobin immunoassay)

Slides were non-covalently coated with capture anti-Myoglobin Ab. First slides were dried on air and covered with the tape containing punched holes (regular size hole puncher) to form wells on the surface of the slides. Coating solution of anti-Myoglobin IgG (50 $\mu\text{g}/\text{mL}$ dissolved in Na-phosphate buffer, 50 mM, pH 7.4) was added to each well (25 μL per well), and slides were incubated for 2–4 hours at room temperature in humid chamber. Slides then were rinsed with water, washing solution (0.05% Tween-20 in water), and water. Blocking was performed by adding blocking solution (1% BSA, 1% sucrose, 0.05% NaN_3 , 0.05% Tween-20 in 50 mM Tris-HCl buffer, pH 7.4; 35 μL per well) and incubation at room temperature for 2–4 hours (or overnight at $+4^\circ\text{C}$) in humid chamber. Slides were rinsed with water, washing solution (0.05% Tween-20 in water), and water. Myoglobin antigen was added at various concentrations (0 to 1000 ng/mL, dissolved in blocking buffer, 25 μL per well) and slides were incubated at room temperature for 1–2 hours and washed as described above, then used for end-point or kinetic measurements.

2.5. End-point binding experiment (model immunoassay)

Dye-labeled conjugate dye-anti-Rabbit IgG (diluted to 10 $\mu\text{g}/\text{mL}$ with Na-phosphate buffer, 50 mM, pH 7.4) was added to ‘sample’ slide (coated with rabbit IgG) or ‘control’ slide (coated with goat IgG) (25 μL per well) and slides were incubated at room temperature in humid chamber for 1–2 hours. Slides then were rinsed with water, washing solution (0.05% Tween-20 in water), and water, and coated with blocking buffer and stored at $+4^\circ\text{C}$ before measurement. Scheme of the model immunoassay is presented in Figure 2.

2.6. End-point binding experiment (Myoglobin immunoassay)

Dye-labeled conjugate dye-anti-Myoglobin Ab (diluted to 10 $\mu\text{g}/\text{mL}$ with Na-phosphate buffer, 50 mM, pH 7.4) was added to the slides coated with anti-Myoglobin Ab and Myoglobin antigen (as described above) at 25 μL per well, and incubated at room

temperature in humid chamber for 1–2 hours. Slides then were rinsed with water, washing solution (0.05% Tween-20 in water), and water, coated with blocking buffer and stored at +4°C before measurement. Scheme of the Myoglobin sandwich immunoassay is presented in Figure 3.

2.7. Detection measurements

Emission spectra in solution were measured using a “Varian Cary Eclipse” fluorometer (“Varian Analytical Instruments”, USA). Absorption spectra in solution and of the surface of the slides were measured using a Hewlett Packard model 8543 spectrophotometer. Fluorescence measurements of the samples on glass slides were performed by placing the slides horizontally on the total internal reflection (TIR) stage as shown on the Figure 4. For excitation we used small solid state laser 532 nm or 651 nm laser diode (commercial laser pointer). Emission spectra were collected by fiber optics from the top using Fiber Optics Spectrometer (SD2000) from Ocean Optics, Inc. For observation we used 550 nm cut-off plastic filter to attenuate excitation line.

3. RESULTS AND DISCUSSION

Our device for the measurement of the fluorescence signal (in total internal reflection mode) from the samples is shown in Figure 4. The sample slide was placed horizontally on the prism, and the signal (full spectrum) detected by the fiber from the top of the well. Most of the immunoassay trial experiments, such as variation of the thickness of the SIF and using different fluorescent labels, were performed using a model immunoassay format (Figure 2). Myoglobin immunoassay (sandwich format, Figure 3) was chosen as an example of cardiac markers, and tested on SIF using optimal SIF thickness and two different fluorescent labels.

3.1. Immunoassay Specificity

We tested the specificity of our assay configuration using model immunoassay - Tetramethylrhodamine-anti-Rabbit IgG binding to the antigen (Rabbit IgG) immobilized on the substrate surface, glass slide, or SIF-coated glass slide. Control slides were coated with a “wrong” antigen, Goat IgG. Thus, the signal from control (Goat) slides shows the non-specific binding of the Tetramethylrhodamine-anti-Rabbit IgG conjugate. The respective emission spectra on SIF-coated and non-coated (glass) slide are shown on Figure 5. For both, SIF and glass, the background was visible; however, its level was much lower than respective specific signal.

3.2. Enhancement of the signal from the SIF versus glass

We tested several fluorescent labels to check the enhancement of the signal for the model immunoassay detected from SIF versus glass slide. Figure 6 shows a typical example: the emission spectra of Alexa Fluor-647 labeled anti-Rabbit IgG bound to the antigen (Rabbit IgG) on SIF and glass. Depending on the particular silver-coated slide, we observe about 10-fold increase in intensity from the silver coated part of the slide, as compared with the unsilvered part of the slide. Similar signal enhancements were obtained for different labeling dyes.

Due to the non-homogeneous slide surfaces and variations in the slide thickness, we observed variations in the enhancement ratio as well. Selected example for high signal variation for Tetramethylrhodamine-anti-Rabbit conjugate is shown on Figure 7.

We varied slide thickness by changing the SIF forming time, and tried to estimate optimal SIF (SIF slide absorbance). Enhancement ratios (SIF signal to glass signal) at different slides are presented in Figure 8. Due to large deviations, a clear conclusion about the optimal SIF slide absorbance cannot be made; however, absorbance should be more than 0.3 OD, and optimal absorbance interval is approximately 0.5 to 0.8 OD (Figure 8).

Enhancement ratio depends on the type of the fluorescent label used, on the labeling degree (dye/protein ratio), and on the excitation wavelength. Table 1 presents a summary of the enhancement data for the model immunoassay using various fluorescent labels and excitation wavelength. No clear conclusions could be made on the optimal type of the label. Dye/protein ratio should be about 3–4 rather than 1–2. The enhancement appears higher when exciting at longer wavelength, but this conclusion should be considered only as preliminary (due to large deviations in this case) and requires further investigations.

3.3. Kinetics of binding

The important characteristic of the immunoassay is kinetics of binding. Figure 9 shows the binding kinetics for Rhodamin Red-X labeled anti-Myoglobin antibody to the Myoglobin bound to the capture antibody on the silver island film surface. The kinetics was monitored starting immediately after addition of the labeled Ab solution to the well. For the first 0.5 min the signal increase is very fast; within about 3 min the signal approaches the plateau, and later, next 10–15 minutes, only a slight increase can be monitored.

Similar kinetics was observed also for the model immunoassay using various labels. The plateau was reached in this case not in 3 min, but in about 10–15 min. When a wrong antigen was used for the model immunoassay (non-specific binding of labeled anti-Rabbit Ab to the Goat IgG antigen), no significant increase of the signal was observed within the first 30 minutes.

3.4. Myoglobin Immunoassay on SIF: end-point and kinetic approach

Next we tested the effect of Myoglobin concentration on the measured signal in the end-point assay experiment. The samples of different Myoglobin concentrations were prepared as described in Methodology part. Figure 10 shows detected signal for silver (SIF) and glass. Although the assay was not optimized according to the antibody pairs and incubation times neither for SIF nor for glass surface, we can see that the sensitivity is improved on SIF surface as compared to glass. The signal for a Myoglobin concentration of 100 ng/nL (which is the clinical cut-off value for Myoglobin 11) on SIF is already higher than the background signal at 0 ng/nL (Figure 10). For glass a concentration of 500 ng/nL is still comparable to the background.

Kinetic approach allows the improvement of the Myoglobin immunoassay on SIF. Fluorescence signals for different Myoglobin concentrations measured in the end-point and kinetic modes are shown on Figure 11. The kinetic data were calculated as the initial

reaction rate (linear approach) within the first 0.5 min. The kinetic set of experimental data shows much lower contribution of background signal. It is probably because the non-specific binding occurs with much slower rate, and fluorescent background grows much slower. At the kinetic mode the sufficient signal level is obtained with the concentrations much below 100 ng/nL.

5. CONCLUSIONS

In this report we present our first results that show high potential of metal enhanced fluorescence for developing an universal platform for cardiac markers detection. The initial results for Myoglobin shows that it is possible to detect Myoglobin concentrations below 50 ng/nL which is lower than clinical cut-off for Myoglobin in healthy patients.

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References

1. Panteghini M. The measurement of cardiac markers: where should we focus? *Am J Clin Pathol.* 2002; 118:354–361. [PubMed: 12219777]
2. Malasky BR, Alpert JS. Diagnosis of myocardial injury by biochemical markers: problems and promises. *Cardiol Rev.* 2002; 10:306–317. [PubMed: 12215194]
3. Fesmire FM. Improved identification of acute coronary syndromes with delta cardiac serum marker measurements during the emergency department evaluation of chest pain patients. *Cardiovasc Toxicol.* 2001; 1:117–123. [PubMed: 12213983]
4. McCord J, Nowak RM, Hudson MP, McCullough PA, Tomlanovich MC, Jacobsen G, Tokarski G, Khoury N, Weaver WD. The prognostic significance of serial myoglobin, troponin I, and creatine kinase-MB measurements in patients evaluated in the emergency department for acute coronary syndrome. *Ann Emerg Med.* 2003; 42:343–350. [PubMed: 12944886]
5. Gosling JP. A decade of development in immunoassay methodology. *Clin Chem.* 1980; 36:14808–1427.
6. Hemmila, IA. Applications of Fluorescence in Immunoassays. John Wiley & Sons; New York: 1992.
7. Vo-Dinh T, Sepaniak MJ, Griffin GD, Alarie JP. Immunosensors: Principles and applications. *Immunomethods.* 1993; 3:85–92.
8. Dickson EF, Pollak A, Diamandis EP. Ultrasensitive bioanalytical assays using time-resolved fluorescence detection. *Pharmacol Ther.* 1995; 66:207–235. [PubMed: 7667396]
9. Lakowicz JR. Radiative decay engineering: biophysical and biomedical applications. *Anal Biochem.* 2001; 298:1–24. [PubMed: 11673890]
10. Lakowicz JR, Shen Y, D'Auria S, Malicka J, Gryczynski Z, Gryczynski I. Radiative decay engineering 2: effects of silver island films on fluorescence intensity lifetimes and resonance energy transfer. *Anal Biochem.* 2002; 301:261–277. [PubMed: 11814297]
11. Castaldo AM, Ercolini P, Forino F, Basevi A, Vrenna L, Castaldo P, D'Ambrosio V, Castaldo A. Plasma myoglobin in the early diagnosis of acute myocardial infarction. *Eur J Clin Chem Clin Biochem.* 1994; 32:349–53. [PubMed: 8086517]

Figure 1.
Typical absorbance spectrum of the SIF-coated glass slide.

Figure 2.
Scheme of the model immunoassay.

Figure 3.
Scheme of the Myoglobin sandwich immunoassay.

Figure 4.
TIR measurement platform.

Figure 5.

Fluorescence spectra of bound tetramethyl-rhodamine-anti-Rabbit IgG conjugate taken from surface of SIF-coated slide and glass slide (average of 2 slides, 6 spots per slide for each spectrum); white circles – SIF; white triangles – glass; grey circles – SIF-control; grey triangles – glass-control.

Figure 6.

Fluorescence spectra (at excitation 532 nm) of Alexa Fluor-647-anti-Rabbit IgG bound to the antigen (Rabbit IgG) immobilized on the SIF substrate (black) or glass substrate (grey).

Figure 7. Fluorescence level at maximum of emission of bound Tetramethylrhodamine-anti-Rabbit conjugate taken from surface of SIF-coated slide and glass slide (different lots of slides prepared in separate experiments shown; average of 6 spots per lot; error bars show one SD).

Figure 8.
Variations in the fluorescence signal ratio SIF/glass of the TRITC-anti-Rabbit antibody bound to the antigen (Rabbit IgG) immobilized on SIF, versus SIF absorbance.

Figure 9.

Kinetics of binding of Rhodamin Red-X labeled anti-Myoglobin Ab to Myoglobin on SIF on glass ([Myoglobin] = 200 ng/mL, excitation 532 nm).

Figure 10. Fluorescence of Alexa Fluor-647 labeled anti-Myoglobin Ab at different Myoglobin concentrations on SIF on glass (grey); white dotted – glass only (excitation 532 nm).

Figure 11.

Fluorescence of Rhodamin Red-X labeled anti-Myoglobin Ab at different Myoglobin concentrations on SIF on glass (excitation 532 nm); A – end-point measurements; B – kinetic measurements (measured within first 0.5 min of the binding).

Table 1

Fluorescence of labeled with different dyes anti-Rabbit IgG bound to the antigen (Rabbit IgG) immobilized on the SIF substrate or glass substrate.

excited at 532 nm	SD SIF %	SD glass %	dye/IgG, mol/mol	measured at	RATIO
TRITC(MolProbes)	12%	36%	3.9	579nm	9.6
TRITC(Sigma)	25%	34%	1.4	575nm	6.6
Rhodamine Red X (MolProbes)	18%	22%	3.8	592nm	4.6
AlexaFluor647 (MolProbes)	21%	38%	4.5	674nm	7.6
AlexaFluor680 (MolProbes)	23%	126%	3.2	705nm	7.0
AlexaFluor750 (MolProbes)	15%	21%	3	582nm	4.0
<i>excited at 651 nm</i>	SD SIF %	SD glass %	dye/IgG	measured at	RATIO
AlexaFluor647 (MolProbes)	31%	51%	4.5	677nm	15.8
AlexaFluor680 (MolProbes)	27%	24%	3.2	708nm	62.2
AlexaFluor750 (MolProbes)	40%	26%	3	773nm	33.1
<i>excited at 663 nm (fluorometer)</i>	SD SIF %	SD glass %	dye/IgG	measured at	RATIO
AlexaFluor680 (MolProbes)			3.2	707nm	8.5