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Original Research

Increase in the detection sensitivity of a lateral flow assay for a cardiac marker by oriented immobilization of antibody

Yiseul Ryu[1], Zongwen Jin[1], Mun Suk Kang[2] & Hak-Sung Kim[1,3]

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Abstract Early detection of human cardiac markers is regarded as a gold standard for diagnosis of acute myocardial infarction (AMI). Here we demonstrate the increase in the detection sensitivity of a lateral flow assay for a cardiac marker troponin I (cTnI) by oriented immobilization of antibodies on magnetic beads. For the construction of sensitive magnetic labels, orientated-immobilization of anti-troponin I antibodies on magnetic beads was attempted using protein G. Magnetic beads were first conjugated with protein G followed by immobilization of anti-cTnI antibodies. The factors affecting the detection sensitivity in the lateral flow assay system were investigated. The lateral flow assay of cTnI was carried out using the magnetic beads and a Giant magnetoresistive (GMR) sensor at the optimized conditions, and compared the results with using a conventional random immobilization method. Magnetic labels with oriented immobilization of antibodies enabled the detection of cTnI up to 0.01 ng/mL, showing significantly increased sensitivity compared with conventional immobilization method. This result clearly shows that the magnetic labels with oriented immobilization of antibodies can find wide applications in the lateral flow assays of target analytes.

Keywords: Protein G, Lateral flow assay, Oriented immobilization, Magnetic bead, Cardiac Troponin I (cTnI)

[1]Department of Biological Sciences, KAIST, Daejon, Korea
[2]LG Innotek Ltd., Ansan, Korea
[3]Graduate School of Nanoscience and Technology, KAIST, Daejeon, Korea
Correspondence and requests for materials should be addressed to H.-S. Kim (✉hskim76@kaist.ac.kr)

Introduction

Acute myocardial infarction (AMI) is the world's leading cause of mortality. According to the latest data by the American Heart Association (AHA), 8.5 million cases of myocardial infarction in the US were reported in 2006, including 935,000 new and recurrent cases and 141,462 deaths[1]. Since timing of AMI management is extremely critical, early and quick diagnosis of AMI is essential for prevention of AMI-related death. Based on current guidelines, the test of cardiac markers should be completed within 30 min ("vein-to-brain" time) so that treatment can be initiated within 60-90 min ("door-to-needle" time) after the patient has arrived at the emergency room or intensive care unit[2,3]. Common biomarkers in clinical diagnosis include creatine kinase-MB isoenzymes, cardiac myoglobin, and troponins[4]. Of them, cardiac troponins (cTnI, cTnT) are considered as the "gold standard" for diagnosis of AMI due to their specificity for myocardial cell damage[5,6]. Hence, the development and implementation of point-of-care testing (POCT) for cardiac troponins have been of great significance.

Lateral flow assays have attracted much attention as one of the most commercially available devices for POCT. Colloidal gold label is frequently used in lateral flow assays, but it has some limitations in terms of sensitivity and quantitative analysis of a target analyte. Lateral flow assays usually rely on detection by naked eyes, and the detection limit of a target analyte by the gold label is approximately 1 ng/mL in sandwich-type assay[7]. Much effort has been made to increase the detection sensitivity in the lateral flow assays, and different labels such as fluorescence, luminescence and magnetic particles have been attempted. Especially, magnetic label offers an advantage over other meth-

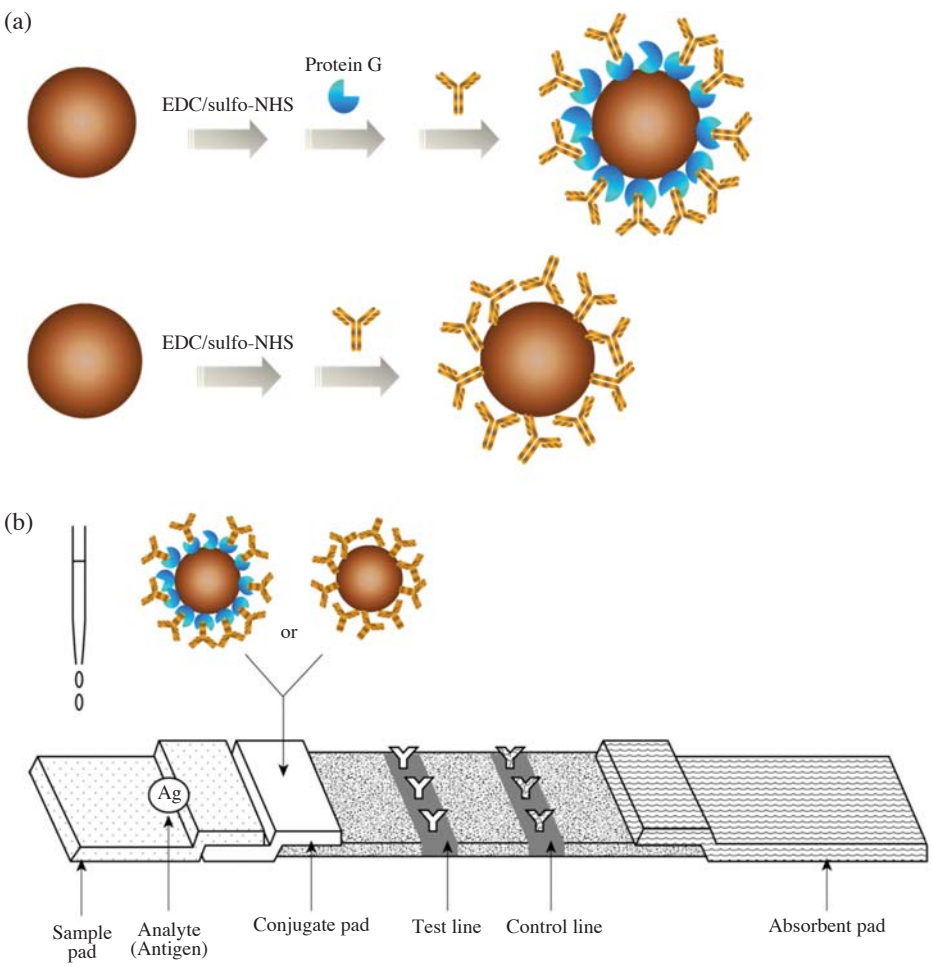
ods. Magnetic labels allow for the detection of the signal from inside membrane, whereas other optical labels are visible only within 10 μm from the surface of membrane due to the opacity of the nitrocellulose membrane. When considering the general depth of lateral flow membrane with at least 100 μm thick, the transparency endows magnetic labels with high detection sensitivity.

Here, we demonstrate an increase in the detection sensitivity of a lateral flow assay for a cardiac marker troponin I (cTnI) by magnetic labels with oriented immobilization of antibodies (Scheme 1). Magnetic beads were first conjugated with protein G through EDC/sulfo-NHS coupling reaction followed by immobilization of anti-cTnI monoclonal antibodies. For comparison, anti-cTnI monoclonal antibodies were directly immobilized onto magnetic beads by coupling with zero-length linker using EDC/sulfo-NHS. Following injection of an analyte-containing sample and premade conjugate solution into the test membrane, the signals from the test and control lines were quantified by a Giant magnetoresistive (GMR) sensor. The signals were linearly correlated with the concentration of troponin I, and the detection sensitivity was estimated to be 0.01 ng/mL for troponin I. Details are reported herein.

Results and Discussion

Construction of a magnetic label by oriented immobilization of antibodies

Immobilization of antibodies on carriers in an oriented manner was shown to be critical for achieving high sensitivity in immunoassays[8-12]. Even though lateral flow assays are widely used, most of labels were constructed by random immobilization of antibodies rather than oriented immobilization. Many approaches for oriented immobilization of antibodies have been re-



Scheme 1. Schematic representation for the lateral flow assays using magnetic labels. (a) Immobilization of antibodies onto magnetic beads in oriented and random manners. (b) Lateral flow assay of a target analyte using magnetic labels.

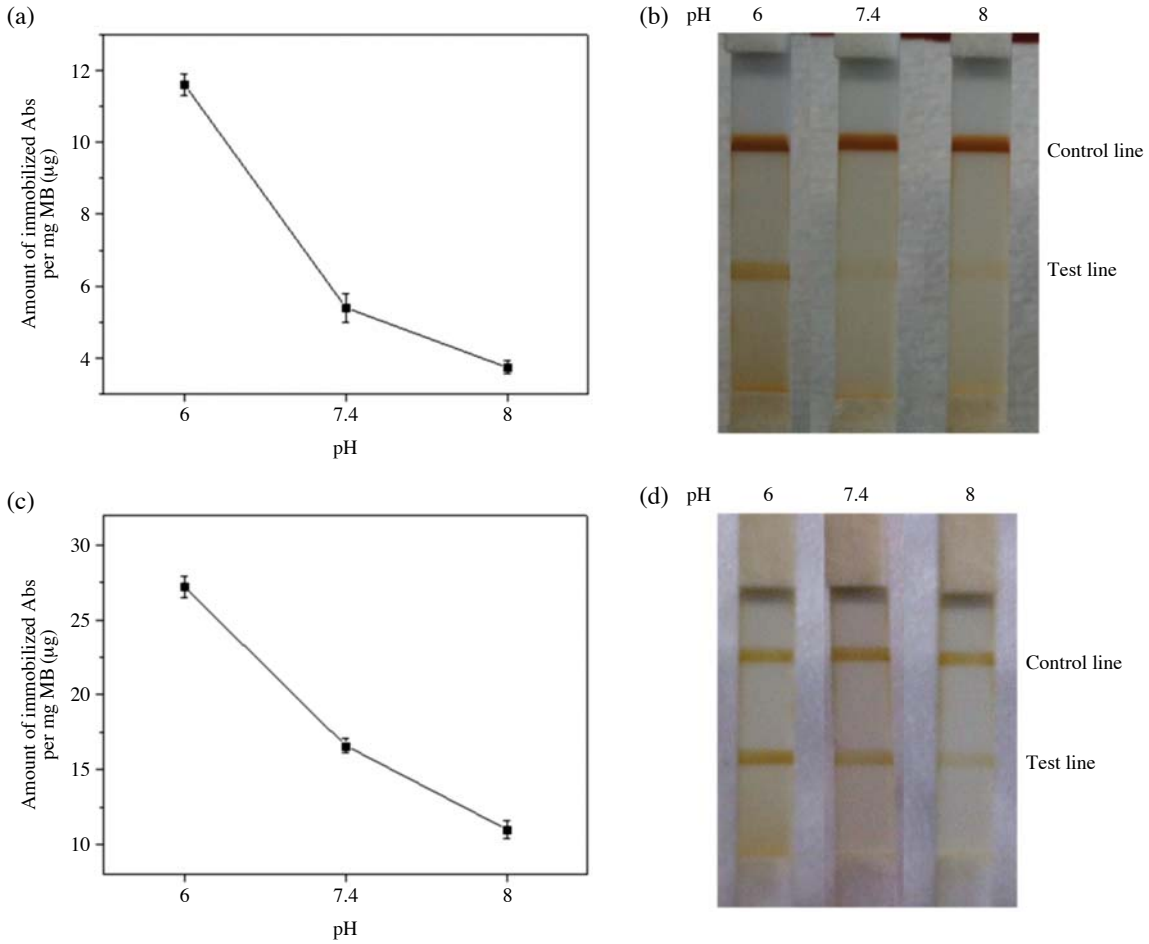


Figure 1. Effects of pH on the amounts of immobilized antibodies and signal intensities in the test lines when antibodies were immobilized by oriented approach (a, b) and random covalent conjugation method (c, d). The concentration of cTnI-C complex used for the assays was 25 ng/mL.

ported, and the use of protein A or G was shown to be effective for oriented immobilization of antibodies[13-16]. Since protein A and G bind only the F_c region of immunoglobulin antibodies, immobilization of antibodies using protein A or G leads antigen binding region (F_{ab}) of antibodies to be exposed towards antigens. It was reported that oriented immobilization of antibodies increased the binding efficacy of a ligand by approximately 2-fold compared with that by random immobilization using amine-coupling reactions[17].

We first attempted to construct magnetic labels for the lateral flow assay to detect troponin I for oriented immobilization of anti-troponin I antibodies on magnetic beads using protein G, magnetic beads were first conjugated with protein G followed by immobilization of anti-troponin I antibodies. Typically, 50 μg protein G was reacted with 1 mg of magnetic beads which had been activated with EDC/sulfo-NHS, and different conjugation times and pH were tested. For immobilization of antibodies, 40 μg of antibody were reacted with 1 mg of protein G-coated magnetic beads. The performance of the constructed magnetic beads were evaluated by the lateral flow assay using Giant magnetoresistive (GMR) sensor. The signal in the test line was the darkest when the conjugation of protein G with magnetic beads was carried out at pH 7.4 for 2 hrs. We also tested the effect of pH on the immobilization of antibodies on protein G-coated magnetic beads by measuring the signal in the lateral flow assays. Most of the bioconjugation reactions were shown to be favorable at pH ranging from 6 to 8, and we also conducted the conjugation within these pH ranges. The amount of bound antibodies dercreased with the increasing pH (Figure 1a), and consequently the signal intensities in the test line of the nitrocellullose membrane became weaker (Figure 1b). About 330 antibody molecules were estimated to be conjugated onto a single magnetic bead when conjugated at pH 6. Based on

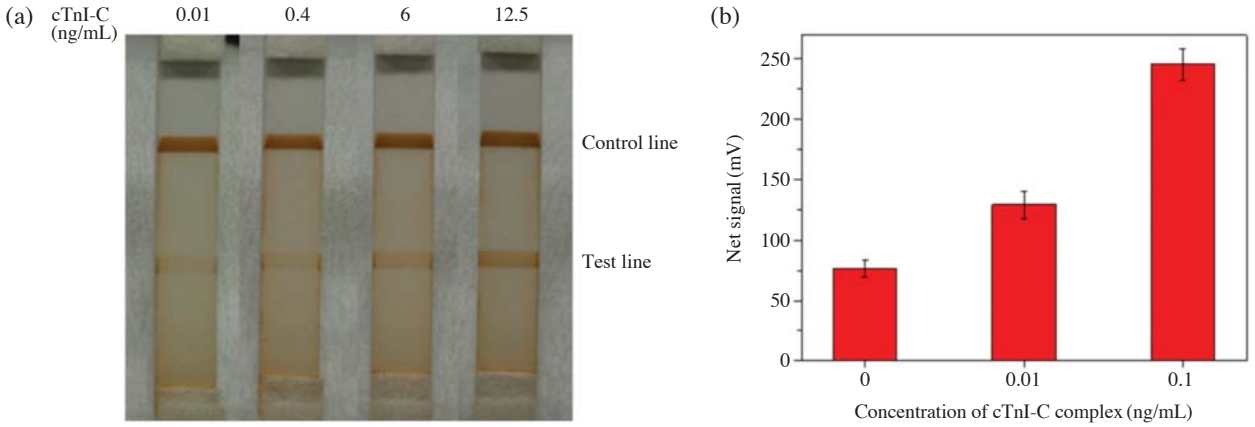


Figure 2. Correlation between the concentration of cTnI-C complex and signal intensity in the lateral flow assays (a). The concentrations of cTnI-C complex tested were 0.01, 0.4, 6, and 12.5 ng/mL. The signals in the test line by a GMR sensor when the concentrations of cTnI-C complex were 0, 0.01, and 0.1 ng/mL (b). Bars indicate standard deviations (n=5).

the result, we conjugated protein G on magnetic beads at pH 7.4 for 2 hrs, whereas immobilization of antibodies onto protein G-coated magnetic beads was carried out at pH 6. To minimize the non-specific protein binding onto magnetic beads, the surface of magnetic beads were passivated with blocking agents. We tested different kinds of blocking agents and found that 0.05% of bovine serum albumin (BSA) is most effective for minimizing the non-specific protein binding.

For comparison, random immobilization of antibodies onto magnetic beads was also carried out. We tested the effects of immobilization conditions on the amount of bound antibodies and the signal intensity in the lateral flow assay. Similarly, the amount of bound antibodies decreased with the increasing pH (Figure 1c), and the signal intensities in the lateral flow assays also decreased (Figure 1d). About 900 antibody molecules were estimated to be conjugated onto a magnetic bead at pH 6, which is about 3-fold higher than that by oriented immobilization. The amount of bound antibodies increased with the increasing concentration of added antibodies, and highest level was observed when 80 μg of antibody was added. But, the highest signal intensity in the lateral flow assays was observed when 40 μg of antibody was added.

Lateral flow assay using magnetic labels with oriented immobilization of antibodies

We performed the lateral flow assays of cTnI-C complex using magnetic beads with oriented immobilization of antibodies by GMR sensor, and determined the detection sensitivity. Sample solution of cTnI-C complex in the range of 0.01, 0.4, 6, 12.5 ng/mL was used for the assays. The signal intensities increased gradually with the increasing concentration of cTnI-C complex (Figure 2a). To determine the detection sensitivity, different concentrations of cTnI-C complex were loaded to the membrane strips, and the signals were measured by a GMR sensor. As shown in Figure 2b, the signal intensities linearly increased with the increasing cTnI-C complex concentration, and the signal to noise ratios were estimated to be 1.68 and 3.20 for 0.01 and 0.1 ng/mL of cTnI-C complex, respectively. The detection sensitivity in the lateral flow assays using magnetic beads with oriented immobilization of antibodies was determined to be about 0.01 ng/mL. On the other hand, when magnetic beads with randomly immobilized antibodies were used, no significant differences in the signal intensity were observed even though the cTnI-C complex concentration increased. This result demonstrates that oriented immobilization of antibodies on magnetic beads provides more antigen-binding regions, making a major contribution to high detection sensitivity even though the amount of immobilized antibodies was a third of randomly conjugated antibodies. The levels of disease biomarkers are very low in blood, and higher sensitivity in the assays is strongly required in diagnosis. Our result clearly shows the increase in the detection sensitivity of a cardiac troponin I (cTnI) in the lateral flow assays by oriented immobilization of antibodies on magnetic beads. We believe that the present approach can find wide applications in the lateral flow assays of target analytes.

Materials and Methods

Reagents and apparatus

MES (2-[*N*-morpholino] ethane sulfonic acid), BSA (Albumin, bovine serum, Fraction V, Approx. 99%),

Tween 20, Phosphate buffered saline (PBS) were purchased from Sigma (USA). Sodium phosphates (monosodium salt and sodium phosphate, di-sodium salt) were from Junsei (Japan). EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), sulfo-NHS (*N*-Hydroxysuccinimide), recombinant Protein G, Bovine Gamma Globulin (BGG) were from Pierce (USA). Bio-Rad protein assay (Bradford solution) was purchased from Bio-Rad (USA). Nunc 96 MicroWell™ Plate was purchased from Nunc (Denmark). Ultra pure water, 18 MΩ cm at 25°C, was used for preparation of solutions. Assays of antibody and magnetic bead were performed using microplate reader (Infinite M200 Megellan, Tecan). Magnetic beads with carboxyl groups (200-nm in diameter) were supplied by LG Innotek Ltd (Korea). The bead consisted of iron oxide particles coated with polystyrene. Two anti-human cTnI monoclonal antibodies (16A11- for conjugating to the magnetic bead, 19C7- for dispensing to nitrocellulose membrane), which are recommended to be best pair for quantitative sandwich immunoassay, were purchased from Hytest (Finland). Troponin complex (I-C), which was used as a standard cardiac marker, was also from Hytest (Finland)[8]. All membrane strips for the lateral flow assays were supplied by LG Innotek Ltd.

Construction of magnetic labels

For oriented immobilization of antibodies onto magnetic beads, protein G was first conjugated to the magnetic beads by EDC/sulfo-NHS. Briefly, 1 mg of magnetic beads were activated by 10 mM EDC and 25 mM sulfo-NHS in 0.1 M MES buffer (pH 6) for 30 min. The resulting beads were then incubated with 50 μg protein G in 0.1 M phosphate buffer (pH 7.4) for 2 hrs under rotator at room temperature. Following washing with 0.1 M phosphate buffer three times, 40 μg anti-cTnI mAb (16A11) was reacted with protein G-coated magnetic beads under rotator for overnight at 4°C. Following a washing with phosphate buffer twice, surface of magnetic beads was blocked with 0.05% (w/v) BSA in 40 mM glycine for 30 min at room temperature. For random conjugation of antibodies, coupling based on EDC/sulfo-NHS reaction was used. Briefly, 1 mg of magnetic beads was activated by 10 mM EDC and 25 mM sulfo-NHS in 0.1 M MES buffer (pH 6) for 30 min. The resulting magnetic beads were then incubated with 40 μg anti-cTnI mAb (16A11) in 0.1 M phosphate buffer (pH 6) for overnight under rotator at room temperature. Following a washing with phosphate buffer twice, surface of magnetic beads was blocked with 0.05% (w/v) BSA in 40 mM glycine for 30 min at room temperature. The magnetic beads conjugated with antibodies were washed with 0.1 M phosphate buffer three times and stored at 4°C.

Quantitative analysis of antibodies and magnetic beads

The amount of bound antibodies on magnetic beads was estimated by measuring the antibody concentrations before and after conjugation reactions. Proteins were assayed by Bradford method. Bovine Gamma Globulin (BGG) was used as a standard for assay of antibodies. Concentrations of magnetic beads were assayed based on the absorption spectra as magnetic nanoparticles[18]. To avoid interference with contamination such as DNA, protein, or salts, magnetic beads were quantified by measuring the absorbance at 420 nm.

Lateral flow assay

Standard cTnI-C complex were adopted as samples in the lateral flow assays. The samples were prepared by diluting the solution of cTnI-C complex in PBS (pH 7.4). For routine assays, 50 μL cTnI-C sample was mixed with 15 μL solution of magnetic bead conjugates and 10 μL 10% (v/v) Tween-20 in 96-well plate. Membrane strips were vertically dipped into a well. After the solution in the well was sucked through the membrane and the top of the membrane was wet, the membrane strip was positioned onto the GMR sensor (LG Innotek Ltd., Korea). The amplified differential voltage signal from the sensor bridge was monitored, averaged, and recorded for correlation with the analyte concentration.

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