Enhancing tools in lateral flow assay for improving detection limit and working range

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Abstract. This work is directed to the estimation of the analytical parameters for improved immunochromatographic tests. A combination of 2 approaches has been proposed to decrease the detection limit of troponin T, a biomarker of acute myocardial infarction. The first approach consists of replacing spherical gold nanoparticles, which are common markers in immunochromatography, with alternative markers, such as gold nanoflowers (AuNFs). The second approach consists of the formation of large aggregates in the analytical zone from AuNFs due to the biotin–streptavidin interaction. The improved tests demonstrated a 10-fold lowering of the detection limit (1.2 ng/ml instead of 11.1 ng/ml) and a 3-fold expanding of the working range of the determined concentrations.

1. Introduction

Among modern analytical methods, immunochromatographic analysis (ICA) is the most popular due to its rapidity, easy implementation in out-of-laboratory conditions, and visual assessment of results. ICA combines chromatographic separation of reagents when the sample moves along the test strip and highly specific interaction of antigens with antibodies. The basic principle of ICA lies in the pre-applied reagents on the membrane components of the test strip at several zones and the movement of the liquid along the membranes after the contact of the test strip with the sample along with all following specific interactions [1]. The content of analytes in a sample is characterized by the intensity of the coloration in analytical zones formed by immunoreagents of different specificity. The coloration is estimated visually (qualitatively) or by using a portable detector (quantitatively) [2].

However, ICA is inferior in sensitivity to instrumental methods of analysis, such as enzyme-linked immunosorbent assay, chromatography, etc. For many substances, their detection in extremely low picogram quantities is in demand. In recent years, a number of approaches have been proposed to decrease the detection limits of ICA while maintaining its rapidity and simplicity [3, 4]. However, these developments are characterized mainly in terms of detection limit, whereas other metrological parameters of the assay, such as accuracy and working range, remain poorly studied.

In view of the above, the presented research was aimed at developing highly sensitive immunochromatographic test systems and their metrological characterization. To increase the ICA sensitivity, we used two approaches. Spherical gold nanoparticles, common markers in immunochromatography, are replaced with alternative markers, such as gold nanoflowers (AuNFs) [5]. The second approach is the formation of large aggregates in the analytical zone from the initial particles via interactions of complementary molecules immobilized on their surface [6]. The complexity of the
metrological characteristics of the amplified assays lies in the fact that they integrate a large number of coupled processes, which entails a variability of conditions and the risks of loss of stability and reproducibility of results. We carried out the development and metrological characteristics of the proposed ICA on the example of determining troponin T (TnT), an important biomarker of myocardial infarction for medical diagnostics [7].

2. Results and Discussion

Two test systems for the detection of troponin T have been developed and characterized. The first is a traditional ICA, in which the role of a colorimetric marker is played by spherical gold nanoparticles with a diameter of 10 nm (figure 1A). The second ICA is based on the use of colloidal particles with a complex shape and a larger size (gold nanoflowers) that form three-component aggregates with the participation of the biotin–streptavidin module (figure 1B).

![Figure 1. Schemes of immune complexes in the analytical zone of test strips for traditional ICA (A) and enhanced ICA (B).](image)

2.1. Synthesis of spherical gold nanoparticles and their characterization

To implement the traditional ICA, spherical gold nanoparticles (SpGNs) with a diameter of 10 nm were synthesized as described in [8]. Their size was determined by transmission electron microscopy on a “Jeol-100” instrument (figure 2A,B) and dynamic light scattering on a “Zetasizer Malvern” instrument (figure 2C). SpGNs have a hydrodynamic radius of 20.6 ± 1.7 nm and an actual diameter of 10.8 ± 3.8 nm.

2.2. Common ICA for TnT detection

SpGNs were conjugated with mouse monoclonal antibodies against troponin T (Abs1/TnT). The concentration of antibodies was 10 µg/ml. Glass fiber membrane with the conjugate having \(A_{520} = 2.0\). Using an IsoFlow dispenser, Abs2/TnT antibodies at 2 mg/ml in PBS with 1% bovine serum albumin (BSA) (forming the test zone) and goat anti-mouse immunoglobulins at 1 mg/ml in PBS (forming the control zone) were applied onto the nitrocellulose membrane.

The assay was carried out at room temperature. The teststrip was vertically submerged into a serum sample for 15 min. After that, the assay results were recorded. ICA allows the obtaining of both qualitative and quantitative results (vLoD and LoD). The latter is obtained by a photometric recording of the color intensity. For this purpose, we used an Epson Perfection V600 Photo scanner with the following processing of digital images by TotalLAB software.
Figure 2. Transmission electron micrograph of SpGNs (A), their size distribution (B), and their dynamic light scattering data (C).

Visual limit of detection (vLoD) was defined as a minimum analyte concentration at which a stained band is seen in the test zone. Instrument LoD was defined as an analyte concentration at which the staining intensity of the test zone exceeds the standard deviation by three times for the background staining (the test zone for samples without cTnT).

The analytical characteristics of the test system were obtained after ensuring all the following optimizations:

- selection of the concentration of the detecting conjugate,
- selection of the concentration of specific antibodies in the test zone,
- optimal analysis time,
- chosen sample preparation.

The common ICA system obtained in this way is characterized by a visual LoD of the TnT in the serum equal to 11.1 ng/ml, an instrumental LoD of 3.7 ng/ml, and an operating range of 3.7–100 ng/ml. The standard deviation in the working range does not exceed 14.1% (n=5).

2.3. Synthesis of gold nanoflowers and their characterization

Gold nanoflowers were synthesized by growing spherical gold nanoparticles, which act as nucleation centers [9, 10]. This technique includes the addition of 4 components: chloroauric acid, sodium citrate,
nucleation centers (gold nanoparticles with a diameter of ~ 10 nm), and hydroquinone. As a result of the redox reaction, a bright blue sol is formed (figure 3), which increases the contrast of the staining of the test strip. AuNFs are characterized by an actual diameter of 89.3 ± 6.4 nm (figure 4 A,B) and a hydrodynamic diameter of 99.6 ± 4.7 nm (figure 4C).

Figure 3. Appearance of colloidal gold sols: A – SpGNs; B – AuNFs.

Figure 4. Transmission electron micrograph of AuNFs (A), their size distribution (B), and dynamic light scattering data (C).

2.4. Enhanced test system
Earlier works showed the advantage of AuNFs as a colorimetric marker in ICA [11, 12]. Due to a more contrasting color, larger size, complex structure, and, as a result, a more developed surface and a larger amount of immobilized protein, LoD decreases 3–5 times.

To form intermolecular aggregates, a number of preparations was obtained: biotinylated Abs1/TnT, biotinylated BSA, and their conjugates with AuNFs (AuNFs–Abs1/TnT–biotin; AuNFs–BSA–biotin) and conjugated streptavidin (SpGNs–Stp). The concentrations of antibodies and streptavidin under the
syntheses was 10 µg/ml and BSA–biotin was 50 µg/ml. After incubation at room temperature, 10% of the BSA or the BSA–biotin solution in water was added (v:v = 40:1).

For fabrication of the enhanced system, two types of glass fiber membranes coated with AuNFs and SpGNs conjugates were prepared. The first membrane was coated with the mixture of the AuNFs–Abs1/TnT–biotin (A_{520} = 2.0) and AuNFs–BSA–biotin (A_{520} = 4.0) and the second membrane was coated with the SpGNs–Stp conjugate (A_{520} = 0.5).

The main parameter that affects the assembly of the three-component complex is the amount of streptavidin. Therefore, we ranged the SpGNs–Stp concentration from 0 to 1.1 optical units (figure 5). From the data obtained, it can be seen that the maximum staining of the test zone is achieved when A (SpGNs–Stp) is equal to 0.5.

Based on the previously performed optimizations, an ICA was developed for the determination of TnT in blood serum. Figure 6A shows the appearance of the test strips. As a result of the photometric registration of the color intensity of the test zone, a calibration curve for the dependence of the color intensity of the test zone on the concentration of TnT in the sample was obtained.

As a result of the calibration curve approximation, an equation that we used to determine the analytical characteristics of the test system was (R^2 = 0.9904):
\[ y = 90058 - \left( 89928 \left( 1 + \left( \frac{x}{x_0} \right)^p \right)^{-1} \right). \]

Table 1 shows the parameters of the developed test system in comparison with the traditional analysis.

| Test system type   | vLoD, ng/ml | LoD, ng/ml | Working range, ng/ml | CV, % |
|--------------------|-------------|------------|----------------------|-------|
| SpGNs              | 11.1        | 3.7        | 3.7–100              | 14.1  |
| Stp–biotin AuNFs   | 1.2         | 0.4        | 0.4–100              | 7.5   |

3. Conclusions
The efforts to increase the sensitivity of cTnT determination by the immunochromatographic method made it possible to reduce the detection limit by 10 times and expand the working range by 3 times. The need for a highly sensitive determination of troponins is justified by the course of troponin-free heart attacks and, as a result, the necessity of further correct treatment of patients in these cases [13, 14]. The rapid revealing of low troponin concentrations will enable quick (in-the-field) and correct diagnoses that prevent deaths [15].

We solved the problem of the reproducibility of multicomponent ICA systems by strict adherence to the conditions of the analysis and the registration of its results. The high reproducibility of the results mainly comes from a correctly selected ratio of biotin–streptavidin, which ensures the unification of the assembly processes of three-component aggregates.

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