A Novel Immunosensing Method Based on the Capture and Enzymatic Release of Sandwich-Type Covalently Conjugated Thionine–Gold Nanoparticles as a New Fluorescence Label Used for Ultrasensitive Detection of Hepatitis B Virus Surface Antigen

Zhaleh Ghafary,† Rahman Hallaj,‡,†,‡ Abdollah Salimi,†,‡,§ and Keivan Akhtari§

†Department of Chemistry, ‡Nanotechnology Research Center, and §Department of Physics, University of Kurdistan, P.O. Box 416, Sanandaj 6617715175, Iran

ABSTRACT: A novel ultrasensitive and simple amplified immunosensing strategy is designed based on a surface-enhanced fluorescence (SEF) nanohybrid made from covalently conjugated thionine–gold nanoparticles (GNP–Th), as a novel amplified fluorescence label, and magnetic nanoparticles (MNPs), as a biological carrier, used for hepatitis B virus surface antigen (HBsAg) detection. This immunosensing strategy operates on the basis of the capture and then release of the amplified fluorescence label. Capturing of the antiHBs-antibody (Ab)-modified GNP–thionine hybrid (GNP–Th-Ab) is carried out through the formation of a two-dimensional (sandwich) probe between this amplified label and antiHBs-antibody-modified magnetic nanoparticles (MNP-Ab), in the presence of a target antigen and using an external magnetic force. Afterward, releasing of the captured fluorescence label is performed using a protease enzyme (pepsin) by a digestion mechanism of grafted antibodies on the GNP–thionine hybrid. As a result of antibody digestion, the amplified fluorescent hybrids (labels) are released into the solution. To understand the mechanism of enhanced fluorescence, the nature of the interaction between thionine and gold nanoparticles is studied using the B3LYP density functional method. In such a methodology, several new mechanisms and structures are used simultaneously, including a SEF-based metal nanoparticle–organic dye hybrid, dual signal amplification in a two-dimensional probe between the GNP–thionine hybrid and MNPs, and a novel releasing method using protease enzymes. These factors improve the sensitivity and speed, along with the simplicity of the procedure. Under optimal conditions, the fluorescence signal increases with the increment of HBs antigen concentration in the linear dynamic range of $4.6 \times 10^{-9}$ to 0.012 ng/mL with a detection limit (LOD) of $4.6 \times 10^{-9}$ ng/mL. The proposed immunosensor has great potential in developing ultrasensitive and rapid diagnostic platforms.

1. INTRODUCTION

Infection by hepatitis B virus (HBV) is an important global public health problem, with significant morbidity and mortality. Even with universal vaccination programs, it has been impossible to significantly prevent acute cases of HBV infection, especially in high-risk populations. Approximately 240 million people are chronic HBV surface antigen (HBsAg) carriers.1,2 Therefore, by developing early diagnostic methods using these proteins, serious injuries can be prevented. Up to now, different methods have been reported for HBV detection, including enzyme-linked immunosorbent assay (ELISA), real-time PCR, electrochemical assay, chemiluminescence assay, fluorescence immunoassay, etc.3−12 But these methods often have some limitations, such as complex processes and equipment and longtime analysis. Therefore, the development of simple, fast, and sensitive sensors can be very advantageous. Up to now, due to their simplicity and stability, antibody–antigen interactions have created a long history in the development of immunosensors, and they have been used in various methods, such as fluorescence immunoassay, colorimetric immunoassay, electrochemical immunoassay, electrochemiluminescence-based immunoassay, SPR-based immunoassay, etc.13−15 To develop more desirable sensors/biosensors, in terms of sensitivity and cost, a basic knowledge of different aspects of science, such as chemistry, biology, nanoscience, and physics, is needed. Up to now, sandwich and competitive assays are the two main strategies used in immunosensors, and among them, the sandwich format is more conventional. In this report, a novel strategy has been put forward based on the sandwich format. This immunosensing strategy operates on the basis of capturing and then releasing of an amplified label. Capturing is carried out through the formation of a magnetic nanoparticle-antibody (MNP-Ab)
and an antibody-fluorescence label (Ab-Label) in the presence of a target antigen to form an MNP-Ab-Ag-Ab-Label, using an external magnetic force. Then the releasing was performed using a protease enzyme (pepsin) by a digestion mechanism of grafted antibodies on the complex. As a result of antibody digestion, amplified fluorescent hybrids are released into the solution. Due to their biocompatibility, ease of functionalization, and paramagnetic nature, magnetic nanoparticles have become an effective part of the most recent reported drug delivery systems, separation systems, and biological applications. Combination of magnetic NPs with a signal generator site using different biological molecules, such as DNA, aptamers, and antibodies, is one of the interesting applications of MNPs.16,17

Proteases are enzymes that catalyze the hydrolysis of the peptide and isopeptide bonds that join amino acids within proteins (known as proteolysis). In the past decade, the digestion of antibodies by proteolytic enzymes (proteases) has been used to study their structure. Many diverse structures can be obtained by fragmentation of the different classes of antibodies with different enzymes, or by using the same enzyme and changing the conditions.18–20 Pepsin is of particular interest as it was the first enzyme to be discovered. It is routinely used for the generation of F(ab) fragments from immunoglobulin G (IgG) and also has the ability to cleave the heavy chains near the hinge region. One or more of the disulfide bonds that join the heavy chains in the hinge region are preserved, so the two Fab regions of the antibody remain joined together, yielding a divalent molecule (containing two antibody binding sites), and hence the light chains remain intact and attached to the heavy chain, whereas the Fc fragment is digested into small peptides.21,22 Common applications of these enzymes are in digestion of antibodies to characterize and analyze their components, preparation of collagen for cosmeceutical purposes, assessment of digestibility of proteins in food chemistry, and subculture of viable mammary epithelial cells.23–28 To date, there has been no report on the use of proteolytic enzymes for captured label release in analytical applications.

On the other hand, ultrasensitive detection in analytical methods requires signal amplification strategies. Common amplification strategies in modernized optical detection methods include the combination of biological molecules, such as antibodies, aptamers, and DNA, with magnetic nanoparticles, quantum dots, etc.29,30 But the pathway toward simplicity and higher detection ability needs more attention to the practical aspect. In this area, the use of extraordinary features of plasmonic materials, to create new sensors and biosensors, has attracted the attention of researchers. By utilizing local surface plasmon resonance (LSPR), plasmonic nanostructures have already been developed as signal amplifiers for optical sensors.31–33 The fundamental of surface-enhanced fluorescence (SEF)-based methods is the coupling of the absorption and emission frequency of a fluorophore with the resonance frequency of a plasmonic material.34 In fact, the plasmonic material acts as an optical antenna. Under appropriate conditions, the fluorescence emission of a fluorescent molecule is strengthened by its placement next to the plasmonic material. In the past decade, gold nanoparticles (GNP) with variable radiate properties, such as absorption, scattering, and surface plasmon resonance, have been brought to the forefront of designed sensors/biosensors. Among the various metals that possess the plasmonic property, gold and silver are more active in the VIS and NIR fields. But silver has lower chemical stability and is easily oxidized. These features make gold the perfect option for building SPR-based systems.35–37 In the designing of a SEF sensor, principles which have to be considered are the plasmon reinforced field, the spectral overlapping between metal plasmon absorption and fluorophore emission, and the metal nanostructure—fluorophore distance.38,39 In this work, the proposed fluorophore is thionine. Thionine (3,7-diamino-5-phenothiazinium), a tricyclic heteroaromatic molecule, is one of the most important members of phenothiazine dyes. It has been widely used as a photosensitizer of large-band gap semiconductors, in the study of electron transfer with DNA, in photoinduced inactivation of viruses, in impedance-based biosensors, etc.40–42 But there are limited numbers of reports on the use of thionine in optical sensors, especially fluorescence, and most of them are related to fluorescence quenching of thionine in the presence of plasmonic metal nanoparticles.43–44 In fact, optical characteristics of a GNP and thionine hybrid drastically change with changing NP size and distance between them, and this dependency allows the possibility of further investigation and development of metal–dye nanohybrids.45 Theoretical studies based on quantum mechanics have a great role in the understanding and investigations of conjugated organic molecules to metal nanostructures. Density functional theory (DFT) and the combination of different methods such as time-dependent DFT (TD-DFT) are used as the most useful theoretical tool in modern empirical sciences, which are at the head of applied research.46–48 In a part of this work, a theoretical study of the gold cluster and its influence on the spectroscopic properties of thionine was conducted, and the first-principles method based on density functional theory (DFT) was used to determine the details of the interaction between gold nanoparticles and thionine. On the basis of all above-mentioned facts and taking into account the need for simple and fast methods, the advantages of SEF, using a covalent hybrid of GNP–thionine, and the advantages of magnetic nanoparticles as biological carriers, to create an ultrasensitive two-dimensional biosensor for hepatitis B virus surface antigen, were used. So we designed a capture–release sandwich-type immunosensor using an anti-HBV-antibody-modified GNP–thionine covalent hybrid (SEF-based nanohybrid) (GNP–Th-Ab) to enhance the signal and anti-HBV-antibody-modified magnetic nanoparticles (MNP-Ab) as biological carriers. Magnetic nanoparticles provide the possibility of collecting the resulting sandwich probes in an easy and fast manner. The greater the amount of analyte, the greater the number of GNP–Th-Ab collected with MNPs. In the next step, for the first time in biosensor mode, releasing of captured signaling sites occurs through digestion of antibodies grafted on the nanohybrids by pepsin. The designed immunoassay has high sensitivity and selectivity for HBs virus antigen. Using this methodology, in the presence of trace amounts of the analyte, very high dual signal amplification is obtained. A comparison of the results of this proposed strategy with those of recently reported biosensors for HBs antigen detection demonstrates the higher sensitivity of our design.

2. RESULTS AND DISCUSSION

2.1. Characterization. Fourier-transform infrared (FTIR) spectra were recorded to investigate the surface modifications of magnetic nanoparticles and covalent modification of gold nanoparticles in the GNP–Th covalent hybrid. Figure 1A shows the FTIR spectra of bare Fe3O4 MNP (a) and Fe3O4-3-aminopropyl triethoxysilane (APTES) MNP (b). The observed band at 583 cm−1 is attributed to the presence of Fe–O
stretching vibrations. The silane polymer modification on the surface of magnetite nanoparticles was confirmed by bands at 808 and 1078 cm\(^{-1}\), which are related to the Si–OH and Si–O–Si groups. Also, the bands at 2922 and 2854 cm\(^{-1}\) in the Fe\(_3\)O\(_4\)–APTES MNP spectrum are attributed to the stretching vibration of the C–H bond of the propylamine group, which proves APTES functionalization of MNP. The broad band at 3300–3500 cm\(^{-1}\) is due to −OH stretching vibrations. On the other hand, two broad bands, which appeared at 1635 and 3417 cm\(^{-1}\), are attributed to the N–H stretching vibrations and bending mode of a free −NH\(_2\) group, as a result of amine grafting on MNPs. These results indicate that MNPs are successfully functionalized by SiO\(_2\) and APTES.\(^{49,50}\) Covalently, surface modification of gold nanoparticles with thionine was proved by FTIR spectroscopy. Figure 1B shows the IR spectra of free thionine (a) and a covalent hybrid of GNP−thionine (b). The absorption bands at 1604 and 1634 cm\(^{-1}\) in figure (a) are ascribed to N–H bending vibrations, and the bands at 1500 and 1349 cm\(^{-1}\) are related to the skeletal vibration of the phenyl ring of thionine. The bands at 2732 and 2937 cm\(^{-1}\) are related to N–H stretching vibrations. These peaks are also seen in the case of the GNP−thionine hybrid, which indicates the proper thionine attachment on gold nanoparticles. The absorption bands at 2827 and 2921 cm\(^{-1}\) are related to citrate impurity in the sample. The absorption bands at 3139 and 3360 cm\(^{-1}\) are related to the N–H stretching vibration of the amino groups, as a result of amine grafting on MNPs. These results indicate that MNPs are successfully functionalized by SiO\(_2\) and APTES.\(^{49,50}\) Covalently, surface modification of gold nanoparticles with thionine was proved by FTIR spectroscopy. Figure 1B shows the IR spectra of free thionine (a) and a covalent hybrid of GNP−thionine (b). The absorption bands at 1604 and 1634 cm\(^{-1}\) in figure (a) are ascribed to N–H bending vibrations, and the bands at 1500 and 1349 cm\(^{-1}\) are related to the skeletal vibration of the phenyl ring of thionine. The bands at 2732 and 2937 cm\(^{-1}\) are related to N–H stretching vibrations. These peaks are also seen in the case of the GNP−thionine hybrid, which indicates the proper thionine attachment on gold nanoparticles. The band at 940 cm\(^{-1}\) can be attributed to the C–S bending vibrations of the heteroaromatic ring of thionine, and the band at 856 cm\(^{-1}\) is due to the N–H bending vibration of amino groups, which are very clear in the IR spectra of the GNP−thionine hybrid, confirming the covalent attachment, which indicates the presence of N–H bonds in the vicinity of the gold nanoparticle surface. Due to the electron deficiency of nitrogen and sulfur atoms in the central ring, they tend to have lower binding to the nanoparticles’ surfaces. On the basis of the above fact, it can be concluded that there are free amino groups near the GNP surface, indicating the formation of the covalent GNP−thionine hybrid.\(^{51,52}\) Scanning electron microscopy (SEM) was used to further characterize the prepared nanostructures. The field emission scanning electron microscope (FE-SEM) image of the synthesized Fe\(_3\)O\(_4\) magnetic nanoparticles showed a relatively spherical and uniform morphology with an average diameter of about 30–40 nm, Figure 2A. The SEM image of gold nanoparticles shows a spherical morphology with an average diameter of about 10–15 nm Figure 2B. Finally, the SEM image of the prepared sandwich probe (GNP−Th-Ab-Ag-Ab-MNP) obviously indicates the accumulation of a large number of GNP−Th hybrids around the magnetic nanoparticles, Figure 2C.

2.2. Effect of Physical Adsorption and Covalent Binding of Thionine on SEF. On the basis of the credibility of simulation studies, chemical attachment of thionine onto the GNP enhances the fluorescence intensity. To provide evidence...
Figure 3. Excitation and emission spectra of (a) free thionine, (b) GNP–Th covalent hybrid, and (c) adsorbed Th on GNPs.

and evaluate the performance of this theoretical prediction, two methods, physical adsorption and covalent attachment, were tested for thionine and gold nanoparticle attachment. Figure 3 shows the excitation and emission spectra of free thionine (a), a covalent hybrid of GNP–Th (b), and adsorbed thionine on GNPs (c). The peak at 520 nm is the spectral property of gold nanoparticles with 15–20 nm diameter. The thionine spectrum in water consists of two absorption bands at 598 and 565 nm, which are related to the absorption property of the monomeric and dimeric forms of a thionine molecule, respectively.53,54 The peak at 565 nm, which is related to the dimeric form, is decreased and that of the monomeric form (598 nm) is pronounced in the covalent GNP–Th hybrid spectrum (c). In the noncovalent binding form, due to the electrostatic interaction between the negatively charged GNPs and the positively charged thionine molecules, spatial saturation of the dye on the nanoparticle increases, and the tendency for dipole–dipole interactions between thionine molecules becomes greater, which results in the formation of the dimeric form of thionine molecules, and this leads to a reduction in thionine fluorescence emission.

2.3. Computational Molecular Model. To simulate the adsorption of thionine and its covalent binding through an anchoring group on the gold nanoparticle surface, full geometry optimizations were performed using the B3LYP functional, the standard 6–31G(d,p) basis set for light atoms, and the LanL2DZ basis set for gold atoms to obtain the ground-state geometry (see Figure 4A). The optimization and single-point energy calculations were performed using the polarized continuum model46 implemented in the Gaussian 98 program.47 The charge-transfer lengths and the charge difference densities were calculated using the Multiwfn program.58 The orbital transition contributions were obtained using the GAUSSSUM 2.2 program.57 To investigate the effect of the anchoring group on the radiative behavior of thionine, the eighteenth lowest excited states of the optimized structures were calculated, and the related parameters are gathered in Table 1. It is well known that fluorescence performance of a structure depends on the distribution of electrons and holes in its excited states.56–59 As a measure of electron–hole (e–h) distribution, the charge-transfer length (Δr) is introduced.58 The electronic state transition is considered in the charge transfer if Δr ≥ 2 Å.56 The values in Table 1 show that the fourth excited state of covalently attached thionine exceeds this criterion considerably. The charge difference density plots of electronic transitions are shown in Figure 4B,C, which reveal the local excitation of electrons and holes for all excited states except for S4 in covalently attached thionine. The enhancement of fluorescence activity of covalently attached thionine can be attributed to the direct recombination of an excited e–h pair in this state. In noncovalently attached thionine, the degree of electron transfer from holes is weak which can be considered as a main factor for the lack of fluorescence activity. To clarify the role of the anchoring group in fluorescence activity, a plot of contributing molecular orbitals in transition states is depicted in Figure 5A,B. As can be seen, the HOMO − 3 is mainly concentrated on the anchoring group, whereas the LUMO mainly concentrates on the dye moiety, which provides a long-range charge transfer for the system. In contrast, the symmetrical population of molecular orbitals for the noncovalent system inhibits a long separation of hole–electron pairs.

2.4. Optimization of Measurement Conditions. To obtain this reinforced fluorescence, various parameters need to be optimized due to its significant role in sensing efficiency. The effect of the gold nanoparticle to thionine ratio on fluorescence emission was investigated by experiments carried out using different amounts of thionine in the presence of a constant concentration of gold nanoparticles. Briefly, 500 μL aliquots of prepared amine-terminated modified GNPs were combined with different volumes of thionine solution in phosphate buffer (4.6 mM, pH 7.4) and stirred for 12 h at room temperature. Afterward, all samples were washed with cooled phosphate buffer and used in the construction of GNP–Th-Ab-Ag-Ab-MNP. The results (Figure 6A) indicate that the optimum GNP/
thionine volume ratio was 5/2 (optimized thionine concentration 1.3 mM). The incubation time required for the antibody–antigen interaction was investigated for probe formation. Briefly, 500 μL of prepared GNP–Th-Ab and 500 μL of prepared MNP-Ab were mixed with equal volumes of antigen solution (1.65 × 10⁻⁷ ng/mL) in 1 mL microtubes. The prepared probes were washed and used in the sensing method. As can be seen in Figure 6B, the optimum incubation time is 2 h.

**Figure 4.** (A) Optimized and fine structures of the GNP–Th covalent hybrid and noncovalently adsorbed Th on GNPs. (B) Plot of the electron–hole (e−h) distribution in noncovalently adsorbed thionine, where the holes and electrons are represented in blue and green, respectively. (C) Plot of the electron–hole (e−h) distribution in covalently attached thionine, where the holes and electrons are represented in blue and green, respectively.

**Table 1. Results of the TD-DFT Calculations in the UV–vis Region for the Models**

| system  | state | ΔE   |   | Δr   | major contribution                  |
|---------|-------|------|---|------|-------------------------------------|
| ads     | S₃    | 2.0909 | 0.0691 | 1.960731 | HOMO − 1 → LUMO (97%)               |
|         | S₄    | 2.325  | 0.0694 | 1.522753 | HOMO − 2 → LUMO (90%)              |
|         | S₅    | 2.6839 | 0.0103 | 0.753743 | HOMO − 1 → LUMO + 1 (94%)          |
|         | S₆    | 2.8615 | 0.0129 | 1.481789 | HOMO − 4 → LUMO (54%), HOMO − 3 → LUMO(28%) |
|         | S₇    | 2.9429 | 0.1072 | 1.255331 | HOMO → LUMO + 2 (73%)              |
|         | S₈    | 3.036  | 0.0505 | 0.594336 | HOMO − 2 → LUMO + 1 (65%)          |
|         | S₉    | 2.5065 | 0.1406 | 0.962928 | HOMO − 2 → LUMO (75%), HOMO − 5 → LUMO(23%) |
|         | S₁₀   | 2.5972 | 0.0075 | 10.92753 | HOMO − 3 → LUMO (97%)              |
|         | S₁₁   | 2.6835 | 0.4852 | 0.506723 | HOMO − 5 → LUMO (73%), HOMO − 2 → LUMO(22%) |

| covalent | S₃    | 2.5065 | 0.1406 | 0.962928 | HOMO − 2 → LUMO (75%), HOMO − 5 → LUMO(23%) |
|          | S₄    | 2.5972 | 0.0075 | 10.92753 | HOMO − 3 → LUMO (97%)              |
|          | S₅    | 2.6835 | 0.4852 | 0.506723 | HOMO − 5 → LUMO (73%), HOMO − 2 → LUMO(22%) |

*The oscillator strengths (fₒ ≥ 0.005), the transition energies ΔE (eV), the charge-transfer lengths Δr (Å), and the major molecular orbital contribution to the intramolecular charge transfer.*
In acidic media (pH ~ 5), the IgG hydrolyzed to (Fab')2 and was digested to small fragments of Fc by the pepsin enzyme.21,22 The received signal during the release process is affected by the pepsin concentration. Therefore, a series of experiments were conducted to investigate the effect of pepsin concentration on the immunosensor response. First, the optimum time for degradation of the antibody by pepsin was evaluated. For this purpose, a 1 mL portion of the prepared probe under optimal conditions was washed and exposed to pepsin solution (0.001 g/mL) for different times, and finally, the fluorescence emission of released GNP-Th was measured. Based on the recorded results (Figure 6C), 8 min was chosen as the optimum time for antibody fragmentation by pepsin. In the next step, 1 mL portions of the prepared probe under the optimal conditions (antigen concentration was 1.6 × 10⁻⁷ ng/mL) were washed and exposed to different concentrations of pepsin (pH ~ 5). The recorded fluorescence intensity for different pepsin concentrations under the optimum conditions is depicted in Figure 6D. As can be seen, the maximum signal is achieved when the pepsin concentration is 0.001 g/mL. Thionine has a neutral form at pH 10, a monomeric form at pH values 2–9, and an H-type dimer form (deprotonated form) at pH values less than 2.53,54

Therefore, pH has a variety of effects on the spectral properties of thionine and GNP-Th. For example, the pH affects the probe formation, fluorescence intensity, digestion activity of pepsin, and undesired adsorption of probe components. In the next step, the pH effect on the probe formation was evaluated. The obtained spectra show that the recorded fluorescence intensity in the pH range of 2–9 is almost constant, and the fluorescence intensity goes down for solution pH values lower than 2 or greater than 9. In aqueous medium, water and thionine undergo a hydrogen bond in the ground state. The reduction in the severity of emission at pH values lower than 2 or greater than 9 should be related to the interactions with negatively charged species, such as OH⁻ and Cl⁻. This effect is known as the solvent effect and is described by the Lippert equation.53,54 As predicted by simulation and theoretical studies (section 2), the recorded results indicate that the fluorescence of SEF-based GNT-Th (b) is higher than the fluorescence of free thionine (a) in a wide range of pH values, even in higher thionine concentrations, Figure 6E. Finally, as a considerably effective factor, the concentration of HBV-Ab for the produced signal was optimized. Based on recorded fluorescence emission for a fixed concentration of antigen (3.2 × 10⁻⁶ ng/mL) and various concentrations of HBV-Ab, 2.5 μg/mL was chosen as the optimum concentration (Figure 6F).

**2.5. Fluorescence Signal Amplification in SEF-Based Immunosensors.** To develop immunosensors with ease of preparation and operation, lower cost, and higher sensitivity, a different strategy could be very beneficial. In our proposed method, HBsAg is measured on the basis of the formation of a sandwich immunosensor, using GNP-Th-Ab, HBs antigen

![Figure 5.](https://example.com/figure5.png) (A) Graphical representations of frontier orbitals of noncovalently adsorbed thionine. (B) Graphical representations of frontier orbitals of covalently attached thionine.
(Ag), and MNP-Ab. GNP−Th-Ab acts as the fluorescence signal generator and amplifier site, and MNP-Ab acts as the probe collector site. Actually, for each antigen molecule, a ball of GNP−Th with amplified fluorescence on the basis of surface plasmon resonance is collected with MNPs. Ultimately, the boosted fluorescence signal is associated with HBsAg concentration. As mentioned, after collecting, washing, and exposing the probe to the acidic solution of pepsin, the GNP−Th hybrid was released into the solution. Then, by measuring the fluorescence of the solution under the optimum conditions, the concentration of HBsAg was measured. The performance of the proposed method was evaluated through the signal competition between control and measuring experiments. The measuring experiment was run by mixing GNP−Th-Ab, HBsAg, and MNP-Ab, and the control experiments were performed by mixing HBsAg, Ab-free GNP−Th, and Ab-free MNP. As depicted in Figure 7, a large difference in fluorescence intensity is observed between the control experiment (a) and the measuring one (b).

2.6. Analytical Performance of SEF-Based Immunoassay toward HBsAg. The analytical performance of the proposed immunosensor for HBsAg detection at various concentrations and optimum conditions was evaluated. Figure 6 shows the recorded typical fluorescence of the immunosensor in the presence of different concentrations of HBsAg. Figure 8A shows the immunosensor response (a) and its related calibration diagram (b) in the range from $6.14 \times 10^{-9}(1)$ to $0.012(10)$ ng/mL, and Figure 8B shows the immunosensor response (a) and its related calibration diagram (b) in the range from $4.6 \times 10^{-7}(1)$ to $1.2 \times 10^{-6}(9)$ ng/mL.
As can be seen, the fluorescence signal increases gradually with HBsAg concentration, and there is a linear relationship between the fluorescence signals. The concentration of HBsAg in the dynamic range can be obtained from $4.6 \times 10^{-9}$ to $0.012$ (ng/mL). The regression equation can be written as $y = 62.672 \ln x + 1212.4$ ($R^2 = 0.994$, n = 10) and the detection limit can be estimated to be $4.6 \times 10^{-9}$ ng/mL. The results suggest that the efficacy of the proposed method for ultrasensitive detection of HBsAg is associated with the capture–release technique and enhanced fluorescence, using the GNP–Th covalent hybrid. Therefore, the present techniques provide the possibility of high signal amplification and, consequently, the possibility for further...
improvements in sensitivity. In addition, compared with the methods in recent reports on HBsAg measurements (Table 2), the developed method exhibits better efficiency and higher sensitivity with a lower detection limit.

Table 2. Comparison of LOD and Linear Dynamic Range of Recently Reported Methods for HBsAg

| method                               | LOD (ng/mL) | dynamic linear range (ng/mL) | ref |
|--------------------------------------|-------------|------------------------------|-----|
| enzyme-linked immunosorbent assay (ELISA) | 0.00116     | 0.047–0.380                  | 4   |
| cyclic voltammetry                    | 0.01        | 0.08–10                      | 5   |
| potentiometry                         | 2.3         | 8–1280                       | 6   |
| stripping voltammetry                 | 87          | 0.1–1500                     | 7   |
| electrochemiluminescent immunoassay (ECLIA) | 8 × 10⁻⁷    | 3 × 10⁻⁴–0.3                | 7   |
| immunochromatographic assay (ICA)     | 0.075       | 0.075–0.0048                 | 8   |
| chemiluminescence                     | 0.1         | 1–200                        | 10  |
| solid surface fluorescence            | 6 × 10⁻⁷    | 5 × 10⁻⁴–0.15               | 11  |
| quartz crystal microbalance (QCM)     | 0.0086      | 0.0086–0.00093              | 12  |
| fluorescence                          | 0.015       | 0.045–6.0                    | 13, 14 |
| fluorescence                          | 4.6 × 10⁻⁹  | 4.6 × 10⁻⁹–0.0012           | this work |

2.7. Reproducibility of the Immunosensor. The reproducibility of the proposed immunosensor was examined by using eight probes, which were prepared separately and used to measure the HBsAg in PBS (pH = 7.4) (Figure 9). The relative standard deviation value was 6.5%, which suggests that the reproducibility of the present immunosensor for the detection of HBsAg is acceptable.

2.8. Real Sample Analysis. To evaluate to what extent the proposed immunosensor could be applicable and to validate and, of course, to get proof of the ability of the strategy, the standard addition method was used by spiking the target HBsAg into a diluted human serum sample (Table 3). An acceptable recovery and relative standard deviation could be observed. The obtained results indicate that the designed methodology in this study has good potential to be employed as a platform to detect HBsAg in real serum samples.

3. CONCLUSIONS
In summary, a novel magnetoimmunoassay is developed for determination of HBsAg. The suggested method includes a capture–release mechanism, and also a new fluorescence label (GNP–Th), which amplifies its fluorescence intensity based on the plasmonic effect of gold nanoparticles. Our proposed immunosensor contains a collector site (MNP-Ab), which collects the fluorescence label (GNP–Th) after the formation of a sandwich probe in the presence of HBsAg. Also, for the first time, a proteolytic enzyme is used to release GNP–Th into the solution. As the results show, the recorded fluorescence signal intensity is related to the HBsAg concentration. The immunosensor efficiency is improved using a GNP–Th covalent hybrid under controlled conditions combined with magnetic nanoparticles as biological carriers. Theoretical calculations are used to study the effect of covalent and noncovalent binding modes and their effect on surface plasmon reinforcement. The designed immunosensor has practical benefits, such as simplicity, low cost, a wide dynamic linear range, selectivity, and high sensitivity. The proposed immunoassay has a high ability for HBsAg measurements in the linear dynamic range of

Table 3. Determination of HBsAg in Spiked Human Blood Serum Samples with the Proposed Immunosensor

| added HBsAg (pg/mL) | found HBsAg (pg/mL) | recovery (%) | RSD (%) (n = 4) |
|---------------------|---------------------|--------------|-----------------|
| 0.24                | 0.23                | 95.83        | 3.1             |

Keywords: fluorescence, capture–release immunosensor, surface-enhanced fluorescence, antibody digestion, protease enzyme, pepsin, thionine, hepatitis B virus surface antigen, gold nanoparticle, magnetic nanoparticle.

Figure 9. Reproducibility of the proposed immunosensor.
4.6 × 10^{-9}–0.012 ng/mL and with a detection limit of 4.6 × 10^{-9} ng/mL. In conclusion, this immunosensor can be a powerful tool to detect and measure other diagnostic biomarkers.

4. EXPERIMENTAL SECTION

4.1. Chemicals and General Techniques. Primary antihuman hepatitis B antibody (HBV-Ab) and HBsAg were purchased from Dia.Pro Diagnostic Bioprobes srl. Tetraethylorthosilicate (TEOS), sodium hydroxide (NaOH), ethanol, glutaraldehyde, ferric chloride, ferrous chloride, ammonium hydroxide (25%), toluene, tetrachloroauric acid (HAuCl₄), sodium citrate, cystamine, and all other reagents of analytical grade were from Merck, Sigma, or Aldrich. The phosphate buffer solution was used for all measurements. Experiments were carried out at room temperature, 25 ± 0.1 °C.

4.2. Apparatus and Procedures. All fluorescence experiments were carried out using an LS-55 Perkin-Elmer spectrophotometer driven with Twin lab software. The morphology and dimensions of the nanoparticles were examined using a field emission scanning electron microscope (FE-SEM). To confirm the nanoparticle phase, the nature of the coating, and its bonding on the surface, Fourier transform infrared spectra (FTIR) were recorded between 4000 and 400 cm⁻¹.

4.3. Preparation of GNP–Th and GNP–Th-Ab. The Turkevich method with some modifications was used to synthesize the gold nanoparticles. Firstly, Haucl₃·3H₂O (0.0078 g) was dissolved in 20 mL of distilled water to obtain a 1 mM solution. This solution was transferred to 50 mL Erlenmeyer and then stirred until boiling point. When the solution reached the boiling point, 2 mL of preheated sodium citrate solution (1%) was added all at once. The color of the solution then slowly turned gray and then red wine color. This color change took about 10 min. After reaching ambient temperature, the synthesized GNPs were kept at 4 °C. To prepare covalently attached thionine on the surface of gold nanoparticles, first, the amine substrate was provided by cystamine; 200 mL of cystamine solution (0.0003 M) in phosphate buffer (7.4) was added to the synthesized GNPs (20 mL), and after 1 h, 1 mL of glutaraldehyde solution was added and stirred for 5 min; after incubation at 4 °C for 2 h, 2 mL of thionine solution (1.3 mM) in phosphate buffer (7.4) was added and stirred for 24 h at room temperature. Then, the resulting hybrid was washed several times with cooled phosphate buffer (7.4) and centrifuged to remove the unreacted components. Physical adsorption of thionine on the GNP surface was performed using the following steps: 2 mL of thionine solution in phosphate buffer (4.6 mM) (7.4) was added to 20 mL of prepared GNPs, and then stirred for 12 h using a magnetic stirrer. Afterward, the resulting mixture was centrifuged and washed with cold phosphate buffer to remove the unadsorbed thionine. These two procedures are shown in Figure 10a,b, respectively. The HBV-Ab modified hybrids were prepared using the following procedure: 10 mL of phosphate buffer (0.1 M, 7.4) was added to the resulting washed hybrids followed by addition of 200 μL of glutaraldehyde solution and 150 μL of antibody solution, and this mixture was incubated for 24 h at 4 °C. After this incubation time, the resulting particles were washed with phosphate buffer to remove unreacted components and kept at 4 °C.

4.4. Synthesis of MNP-Ab. First, MNPs were synthesized in an alkaline solution of ferric chloride and ferrous chloride via the standard coprecipitation method reported by Liu with some modifications. Briefly, FeCl₃·6H₂O (2.7 g) and FeCl₂·4H₂O (1.39 g) were dissolved in 100 mL of deoxygenated distilled water at 80°C under N₂ protection and vigorous mechanical stirring, and then 7.5 mL of ammonium hydroxide were added dropwise. The reaction mixture was stirred for 60 min and then cooled to room temperature. Then, the resulting black precipitate was collected using an external magnet, washed...
four times with water, and then dried at room temperature for 48 h. Subsequently, core/shell Fe$_3$O$_4$@silica was prepared according to the Stöber method with some modifications.

Typically, 1 g of the as-synthesized MNPs were dispersed in a mixture of 100 mL of ethanol and 20 mL of distilled water and sonicated for 30 min. Then, 5 mL of 25% ammonia aqueous solution was added with vigorous stirring followed by dropwise addition of 2 mL of TEOS to this solution, and the reaction mixture was stirred for 12 h under N$_2$ protection. The resulting particles were collected with a magnet, washed several times with ethanol and water, and then dried at room temperature. To prepare amine-functionalized MNPs (MNP-APTES), the obtained powder (1 g) was dispersed in methanol (100 mL) and toluene (30 mL) and sonicated for 30 min, followed by dropwise addition of APTES (99%, 1 mL), and the reaction mixture was stirred for 12 h and separated using a magnet.
were added into the microtubes containing the MNP-Ab with phosphate buffer during the incubation time. After 2 h, the obtained sandwich and GNP was added to them and kept at 4 °C. As a measure of glutaraldehyde solution and 50 μL of antibody solution were added to this suspension and kept at 4 °C for 24 h. Afterward, the resulting MNP-Ab were washed to remove unreacted components, and then 2 μL of phosphate buffer (7.4, 0.1 M) was added to them and kept at 4 °C.

4.5. Immunoassay Procedure and HBSAg Measurement. The prepared and washed probe components (MNP-Ab and GNP—Th-Ab) in phosphate buffer (7.4), according to the above instructions, were used as immunosensing materials. Briefly, 100 μL portions of the prepared GNP—Th-Ab and 100 μL portions of MNP-Ab were mixed into 1 mL microtubes. Subsequently, different concentrations of HBSAg solution (pH = 7.4) were added into the microtubes containing the MNP-Ab and GNP—Th-Ab mixture in PBS. The tubes were shaken during the incubation time. After 2 h, the obtained sandwich complex was collected easily by an external magnet and washed with phosphate buffer (pH 7.4, 0.1 M). Then 500 μL of pepsin solution (0.0005 g/mL) in acetic acid (pH 4.7) was added. As a result, antibodies were digested and the GNP—Th hybrid was released into the solution. After a short time (8 min), the fluorescence of the solution was measured (E2: 560 nm). All measurements were carried out at room temperature (Scheme 1).

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: Rhallaj@uok.ac.ir, Rhallaj@yahoo.com.

ORCID

Rahman Hallaj: 0000-0002-7952-7248
Abdollah Salimi: 0000-0003-1137-1854

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the Iranian Nanotechnology Initiative and the Research Office of the University of Kurdistan.

■ REFERENCES

(1) Hu, J.; Cheng, J.; Tang, L.; Hu, Z.; Luo, Y.; Li, Y.; Zhou, T.; Chang, J.; Guo, J. T. Virological Basis for the Cure of Chronic Hepatitis B. ACS Infect. Dis. 2018, 5, 659–674.

(2) Ortega-Prieto, A. M.; Cherry, C.; Gunn, H.; Dorner, M. In Vivo Model Systems for Hepatitis B Virus Research. ACS Infect. Dis. 2018, 5, 683–702.

(3) Wu, Y.; Zeng, L.; Xiong, Y.; LENG, Y.; Wang, H.; Xiong, Y. Fluorescence ELISA based on glucose oxidase-mediated fluorescence quenching of quantum dots for highly sensitive detection of Hepatitis B. Talanta 2018, 181, 258–264.

(4) Chen, Y.; Wang, J.; Liu, Z.; Wu, G. Determination of hepatitis B virus surface antigen in serum with a sandwich immunosay and capillary electrophoresis–electrochemical detection. Anal. Methods 2014, 6, 2484–2489.

(5) Yuan, R.; Tang, D.; Chai, Y.; Zhong, X.; Liu, Y.; DAI, J. Ultrasensitive Potentiometric Immunosensor Based on SA and OCA Techniques for Immobilization of HBAbs with Colloidal Au and Polyvinyl Butyral as Matrices. Langmuir 2004, 20, 7240–7245.

(6) Shen, G.; Zhang, Y. Highly sensitive electrochemical stripping detection of hepatitis B surface antigen based on copper-enhanced gold nanoparticle tags and magnetic nanoparticles. Anal. Chim. Acta 2010, 674, 27–31.

(7) Babamiri, B.; Hallaj, R.; Salimi, A. Ultrasensitive electrochemiluminescence immunosensor for determination of hepatitis B virus surface antigen using CdTe@CdS-PAMAM dendrimer as luminescent labels and Fe3O4 nanoparticles as magnetic beads. Sens. Actuators, B 2018, 254, 551–560.

(8) Shen, J.; Zhou, Y.; Fu, F.; Xu, H.; LV, J.; Xiong, Y.; Wang, A. Immunochromatographic assay for quantitative and sensitive detection of hepatitis B virus surface antigen using highly luminescent quantum dot-beads. Talanta 2015, 142, 145–149.

(9) Xi, Z.; Huang, R.; LI, Z.; HE, N.; Wang, T.; SU, E.; Deng, Y. Selection of HBSAg-Specific DNA Aptamers Based on Carboxylated Magnetic Nanoparticles and Their Application in the Rapid and Simple Detection of Hepatitis B Virus Infection. ACS Appl. Mater. Interfaces 2015, 7, 11215–11223.

(10) Babamiri, B.; Hallaj, R.; Salimi, A. Solid surface fluorescence immunosensor for ultrasensitive detection of hepatitis B virus surface antigen using PAMAM/CdTe@CdS QDs nanoclusters. Methods Appl. Fluoresc. 2016, 8, No. 035013.

(11) Yao, C.; Zhu, T.; Tang, J.; Wu, R.; Chen, Q.; Chen, M.; Zhang, B.; Huang, J.; Fu, W. Hybridization assay of hepatitis B virus by QCM peptide nucleic acid biosensor. Biosens. Bioelectron. 2008, 23, 879–885.

(12) Lu, Q.; Dong, X.; Zhang, R.; Han, X.; Fang, X.; Zhang, Y. A gold nanorods-fluorescent biosensor for the detection of hepatitis B virus DNA based on fluorescence resonance energy transfer. Analyst 2013, 138, 642–650.

(13) Chen, C.; Zhao, J.; Lu, Y.; Sun, J.; Yang, X. Fluorescence Immunaoassay Based on the Phosphate-Triggered Fluorescence Turn-on Detection of Alkaline Phosphatase. Anal. Chem. 2018, 90, 3505–3511.

(14) Han, X.; Zhang, H.; Zheng, J. Ultrasensitive Electrochemical Immunosassay Based on Cargo Release from Nanized PbS Colloidomes. Anal. Chem. 2019, 91, 2224–2320.

(15) Hu, G.; Sheng, W.; Zhang, Y.; Wang, J.; Wu, X. Wang, S. Upconversion Nanoparticles and Monodisperse Magnetic Polystyrene Microsphere Based Fluorescence Immunosassay for the Detection of Sulfasalazine in Animal-Derived Foods. J Agric. Food Chem. 2016, 64, 3908–3915.

(16) Ha, Y.; Ko, S.; Kim, I.; Huang, Y.; Mohanty, K.; Huh, C.; Maynard, J. A. Recent Advances Incorporating Superparamagnetic Nanoparticles into Immunosassays. ACS Appl. Nano Mater. 2018, 1, 512–521.

(17) Kim, C.; Pearson, P. C. Detection of Plasmodium Lactate Dehydrogenase Antigen in Buffer Using Aptamer-Modified Magnetic Microparticles for Capture, Oligonucleotide-Modified Quantum Dots for Detection, and Oligonucleotide-Modified Gold Nanoparticles for Signal Amplification. Bioconjugate Chem. 2017, 28, 2230–2234.

(18) Kazanov, M. D.; Igarashi, Y.; Eroshkin, A. M.; Cieplak, P.; Ratnikov, B.; Zhang, Y.; LI, Z.; Godzik, A.; Osterman, A. L.; Smith, J. W. Structural determinants of limited proteolysis. J. Proteome. Res. 2011, 10, 3642–3651.

(19) Chu, Q.; Diedrich, K. J.; Vaughan, J. M.; Donaldson, C. J.; Nunn, M. F.; Lee, K. F.; Saghatelian, A. HtrA1 Proteolysis of ApoE In Vitro Is Allele Selective. J. Agric. Food Chem. 2018, 66, 11984–11989.

(20) Mureşan, C. I.; Schierhorn, A.; Buttstedt, A. The Fate of Major Royal Jelly Proteins during Proteolytic Digestion in the Human Gastrointestinal Tract. J. Agric. Food Chem. 2018, 66, 4164–4170.

(21) Yang, X.; Naughton, S. X.; Han, Z.; He, M.; Zheng, Y. G.; Terry, A. V. Jr.; Bartlett, M. G. Mass Spectrometric Quantiﬁcation of Tubulin Acetylation from Pepsin-Digested Rat Brain Tissue Using a Novel Stable-Isotope Standard and Capture by Anti-Peptide Antibody (SISCAPA) Method. Anal. Chem. 2018, 90, 2155–2163.

(22) Pan, L. Y.; Salas-Solano, O.; Valliere-Douglass, J. F. Antibody structural integrity of site-speciﬁc antibody-drug conjugates investigated by hydrogen/deuterium exchange mass spectrometry. Anal. Chem. 2015, 87, 5669–5676.
(24) Pang, Y.; Wang, W. H.; Reid, G. E.; Hunt, D. F.; Bruening, M. L. Pepsin-Containing Membranes for Controlled Monoclonal Antibody Digestion Prior to Mass Spectrometry Analysis. *Anal Chem.* 2015, 87, 10942–10949.

(25) Ho, P.; Ede, C.; Chen, Y. Y. Modularly Constructed Synthetic Granzyme B Molecule Enables Interrogation of Intracellular Proteases for Targeted Cytoxicity. *ACS Synth. Biol.* 2017, 6, 1484–1495.

(26) Guerrero, J. L.; O’Malley, M. A.; Daughtery, P. S. Intracellular FRET-Based Screen for Redesigning the Specificity of Secreted Proteases. *ACS Chem. Biol.* 2016, 11, 961–70.

(27) Gulati, P.; Jia, S.; Li, A.; Holding, D. R.; Santra, D.; Rose, D. J. In Vitro Pepsin Digestibility of Cooked Proso Millet (Panicum miliaceum L.) and Related Species from Around the World. *J. Agric. Food Chem.* 2018, 66, 7156–7164.

(28) Hoernstein, S. N. W.; Fode, B.; Wiedemann, G.; Lang, D.; Niederkrüger, H.; Berg, B.; Schaaf, A.; Frischmuth, T.; Schlosser, A.; Decker, E. L.; Reski, R. Host Cell Proteome of Physcomitrella patens Harbors Proteases and Protease Inhibitors under Bioproduction Conditions. *J. Proteome. Res.* 2018, 17, 3749–3760.

(29) Li, Y.; Dai, W.; Lv, X. Yulin Deng. Aptamer-based rolling circle amplification coupled with graphene oxide-based fluorescence resonance energy transfer for sensitive detection of cardiac troponin I. *Anal. Methods* 2018, 10, 1767–1773.

(30) Zhao, Q.; Piao, J.; Peng, W.; Wang, Y.; Zhang, B.; Gong, X.; Chang, J. Simple and Sensitive Quantification of MicroRNAs via PS@I. Resonance Energy Transfer for Sensitive Detection of Cardiac Troponin. *Langmuir* 2016, 32, 4980–4984.

(31) Yang, Z.; Sassa, F.; Hayashi, K. Plant Biomarker Adsorption Behavior of Thionine on Gold Nanoparticles with Different Group on the Charge Transfer/Recombination Dynamics of a Photoactive Layer: Theoretical Study. *J. Phys. Chem. B* 2011, 115, 13405–13413.

(32) Yang, Z.; Sassa, F.; Hayashi, K. A Robot Equipped with a High-Speed LSPR Gas Sensor Module for Collecting Spatial Odor Array. *J. Colloid Interface Sci.* 2018, 520, 66–73.

(33) Lee, J. H.; Park, B. S.; Ghang, H. G.; Song, H.; Yang, S. Y. Nano-Protrusive Gold Nanoparticle-Hybridized Polymer Thin Film as a Sensitive, Multipatternatable, and Antifouling Biosensor Platform. *ACS Appl. Mater. Interfaces* 2016, 8, 70522–70526.

(34) Lee, J. H.; Toy, T. H.; Park, B. S.; Sun, M. Theoretical Investigations of Optical Origins of Fluorescent Graphene Quantum Dots—Graphene Hybrid Materials. *Nanocharacter* 2018, 29, No. 154202.

(35) Ding, Y.; Chen, Z.; Xie, J.; Guo, R. Comparative studies on adsorption behavior of thionine on gold nanoparticles with different sizes. *J. Colloid Interface Sci.* 2008, 327, 243–250.

(36) Ding, Y.; Zhang, X.; Liu, X.; Guo, R. Adsorption Characteristics of Thionine on Gold Nanoparticles. *Langmuir* 2006, 22, 2292–2298.

(37) Cao, S.; Wang, J.; Ma, F.; Sun, M. Charge Transfer Channel in Quantum Dot—Graphene Hybrid Materials. *Nanotechnology* 2018, 29, No. 154202.

(38) Biews, S.; Pramanik, A.; Sarkar, P. Effect of additional donor group on the charge transfer/recombination dynamics of a photoactive layer on the charge transfer/.
organic dye: A quantum mechanical investigation. *Comput. Theor. Chem* 2017, 1103, 38–47.

(60) Kimling, J.; Maier, M.; Okenve, B.; Kotaidis, V.; Ballot, H.; Plech, A. Turkevich method for gold nanoparticle synthesis revisited. *J. Phys. Chem. B* 2006, 110, 15700–15707.

(61) Stober, W.; Fink, A. Controlled growth of monodisperse silica spheres in the micron size range. *J. Colloid Interface Sci.* 1968, 26, 62–69.

(62) Dezfoolinezhad, E.; Ghodrati, K.; Badri, R. Fe3O4@SiO2@polyionene/Br3–core–shell–shell magnetic nanoparticles: a novel catalyst for the synthesis of imidazole derivatives under solvent-free conditions. *New J. Chem.* 2016, 40, 4575–4587.