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4 **Water Dispersion of Magnetic Nanoparticles with Selective Biofunctionality**
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8 **for Enhanced Plasmonic Biosensing**
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14 Zhong Mei¹, Ashish Dhanale¹, Ajithkumar Gangaharan², Dhiraj Kumar Sardar², Liang Tang^{1,*}
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17 ¹ Department of Biomedical Engineering, University of Texas at San Antonio
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20 ² Department of Physics & Astronomy, University of Texas at San Antonio
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24 San Antonio, TX 78249; USA
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31 ***Corresponding author**
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33
34 Liang Tang, PhD
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37 Department of Biomedical Engineering
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40 University of Texas at San Antonio
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44 1 UTSA Circle
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47 San Antonio, TX 78249
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50 Email: liang.tang@utsa.edu
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54 Tel: +01 210-458-6557; Fax: +01 210-458-7007
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Abstract

Magnetic nanoparticles (MNPs) are widely used in biosensing, bioimaging, and drug delivery. However, high quality superparamagnetic nanoparticles with uniform size were usually synthesized by thermal decomposition using organic solvents. To be suitable for biomedical applications, a facile and efficient water dispersion of iron oxide MNPs from solvent using an innovative agent, sodium oleate (NaOL) was described. The monodispersed MNPs (4 and 15 nm respectively) after transfer was biocompatible and stable at a broad temperature range (4~50 °C) over months. More importantly, the NaOL coating allows for surface modification with selective functionality, rendering the aqueous MNPs highly customizable for biofunctionalization. Little effect on the superparamagnetism was observed after the water dispersion. To further evaluate its practical application in biosensing, custom MNPs were prepared for specific cardiac troponin I (cTnI) detection for myocardial infarction diagnosis. Specifically, gold nanorod (GNR) biochip was probed by the MNP-captured cTnI target analyte at varying concentrations. The signal transduction of the GNR sensor is based on the localized surface plasmon resonance (LSPR). The application of the MNPs resulted in a significant enhancement of the plasmonic response of the GNRs. As such, the MNP-mediated LSPR biosensing showed a three times lower sensitivity as compared to the direct cTnI binding without functional MNPs. Computer simulation further elucidated that the enhancement was distance dependent between the MNP and the surface of the nanorod, which corroborated with experimental results.

Keywords: Magnetic nanoparticle; Phase transfer; Gold nanorod biochip; Surface plasmon resonance; Cardiac troponin I; Heart attack diagnosis

1. Introduction

Magnetic nanoparticles (MNPs) have attracted much attention due to their great potentials in biomedical application such as biological tracking, bioseparation, MRI imaging, and drug delivery [1-4]. Conventionally, water dispersed iron oxide MNPs are prepared from co-precipitation of ferrous and ferric ions in sodium hydroxide solution [5]. The disadvantages such as unstable surface composition and non-uniform size of the resulted nanoparticles limited their use in biomedical application. To overcome these problems, high quality iron oxide nanoparticles are usually synthesized in organic solvents by thermal decomposition of iron precursor complex at high temperatures [6]. The resulting MNPs are mono-dispersed with uniform size which is highly tunable from 2-20 nm. However, the solvent-based nanoparticles are not biocompatible. As such, it is highly desirable to transfer the MNPs from organic solvents to water dispersion. To address this issue, silane ligand exchange [7] was applied to modify the surface of ferrite nanoparticles and render water dispersibility with a tunable hydrophilic periphery. However, this exchange process was time-consuming (at least 72 hours). Moreover, the silane layers on the MNPs caused a great loss of magnetization per unit mass of the particles. Another study was reported for a phase-transfer method by using the amphiphilic polymer (PMAO-PEG) as a surface linker [8]. The use of expensive primary amino group terminated poly(ethylene glycol) methyl ether was not cost-effective and the chemistry process of copolymer synthesis was complicated. Cetyltrimethylammonium bromide (CTAB), a cationic surfactant, was also used to transfer hydrophobic nanocrystals to aqueous phase [9]. However, the cytotoxicity of the CTAB coating often leads to biocompatibility issue.

Herein, we demonstrate a facile phase transfer of MNPs from organic solvent upon thermal decomposition synthesis to water solution using sodium oleate (NaOL) as an innovative transfer

agent. Unlike CTAB, NaOL is a biocompatible surfactant during nanoparticle fabrication [10]. Another feature of NaOL is to provide carboxyl terminals on the MNPs. The meaning of this surface modification is two-fold. One is to enable water soluble MNPs and the other is to facilitate a highly adaptable functionality for bioconjugates after the phase transfer. The capability renders our method as a universal preparation of the high quality MNPs with selective terminals to functionalize with fluorescence, oligonucleotides and antibodies. In common, these molecules contain terminal amino-groups which can be effectively linked to the carboxyl groups of the transferred MNPs through EDC-NHS coupling [11, 12].

To explore the practical use in biosensing, the synthesized MNPs were transferred to aqueous solution, followed by functionalization with antibody against a cardiac biomarker (cTnI) as a model system. The as-prepared MNPs were explored in a gold nanorod biochip based on the localized surface plasmon resonance (LSPR). LSPR arising from gold nanorods has been utilized as the optical transduction of specific biological binding [13-15]. The sensing mechanism relies on the sensitive longitudinal SPR shift which is caused by refractive index change in GNR surrounding environment [16]. However, the solution based GNR probe has the intrinsic problems of fluctuating plasmon reading and short storage time due to nanoparticle aggregation. A chip-based LSPR biosensor is a more robust and fashionable platform. In this study, functional GNRs were assembled on glass substrates to construct a nanoarray biochip. Further improvement of the spectral sensitivity was investigated by integrating the NaOL-coated MNPs into the biochip. To further understand the mechanism, COMSOL simulation was performed to elucidate the distance dependence of the sensitivity enhancement.

2. Materials and methods

2.1 Materials

Iron (III) oxide (FeOOH, hydrated, catalyst grade), oleic acid ($\geq 99\%$), 1-octadecene (technical grade, 90%), gold chloride (HAuCl_4 ; 99%), cetyltrimethylammoniumbromide (CTAB), sodium borohydride (NaBH_4 ; 99%), L-ascorbic acid (AA; 99%), silver nitrate (AgNO_3 ; 99%), sodium oleate (NaOL ; $>97\%$), (3-mercaptopropyl)trimethoxysilane (MPTMS), chloroform (anhydrous, $\geq 99\%$) and hexane (anhydrous, 95%) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were from Thermo Scientific (Rochester, NY). Highly purified human cardiac troponin I antigen (cTnI) and the specific murine monoclonal antibody pairs targeting different cTnI epitopes (aa 24–40 and 87–91, respectively) were obtained from Fitzgerald Industries (Acton, MA). Microscopy glass substrates with ITO coating were from Delta Technologies (Loveland, CO). All reagents were used as received without any further treatment.

2.2 Synthesis of high quality Fe_3O_4 magnetic nanoparticles and phase transfer to water dispersion for biofunctionalization

Monodispersed MNPs of 4–15 nm were synthesized using thermal decomposition method with modifications [17]. Generally, a mixture of FeOOH fine power (0.178 g), oleic acid and 1-octadecene (5.0 g) was added to a three-neck flask with condenser and magnetic stir bar. Under the atmosphere of nitrogen gas, the reaction mixture was heated up to 320°C using controllable thermocouple and heating mantle. The solution turned clear black, indicating the formation of nanoparticles. After maintaining at 320°C for 30 min, the resulting solution containing MNPs was rapidly cooled to room temperature, followed by addition of 20 mL ethanol to precipitate the

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4 magnetic nanoparticles. The MNPs were then separated by centrifugation and washed with
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6 hexane and ethanol three times. The as-prepared Fe₃O₄ nanoparticles were finally redispersed in
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8 15 mL of chloroform before further processing. MNPs of varying sizes can be synthesized by
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10 fine tuning of the molar ratio of FeOOH to oleic acid between 1:3 and 1:4 and the reaction time
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12 from 5 to 30 minutes.
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16 To prepare water dispersion of MNPs for biological applications, a 1 mL aliquot of the
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18 aforementioned MNPs in chloroform was added to 5 mL of aqueous NaOL solution (0.1 M). The
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20 two-phase mixture was stirred vigorously to form a deep brown emulsion. The mixture was then
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22 transferred to a rotary evaporation setup to allow evaporation of the organic solvent under
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24 vigorous stirring. After 15 min at 35 °C, the chloroform was completely removed and a clear
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26 aqueous solution was obtained, resulting in water dispersed carboxyl-terminated nanoparticles.
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28 The MNPs are attracted to the sides using magnet while the excess NaOL solution was decanted.
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30 The purified MNPs were then dispersed in 5 mL MilliQ water.
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36 Surface functionalization of MNPs with fluorescence, oligonucleotides or antibodies was
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38 performed using the established EDC/NHS chemistry [11]. Specifically, 500 µL of NaOL
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40 transferred MNPs was added to 100 µL of PBS buffer (10 mM) containing EDC (2 mg/mL) and
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42 NHS (1 mg/mL) to activate the carboxyl group in 30 min. Then 100 µL of antibody solution (e.g.
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44 anti-cTnI) was reacted for 2 hours at room temperature and the resulting mixture was centrifuged
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46 at 8,000 rpm to remove unbound antibodies in the supernatant. The pellets were redispersed in
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48 10 mM PBS to retrieve the antibody-immobilized MNPs. ELISA tests were performed to
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50 confirm the conjugation of the antibody molecules and the high binding activity to target antigen.
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58 *2.3 Preparation and functionalization of gold nanorod bioprobes*

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Gold nanorods were chemically synthesized by a seed-mediated growth method using CTAB and NaOL bisurfactants [10]. Afterwards, GNRs were first purified by centrifugation twice at 10,000 rpm for 20 min. 5 mL MilliQ water was then added to resuspend the solid pellets and centrifuged again at 13,000 rpm for 3 min. The resulting pellets were finally redispersed to make a total of 5 mL concentrated GNR solution.

Surface modification of GNRs with thiolated biological reporters was conducted following our previously reported protocol [18]. Briefly, 20 μ L of Traut's reagent (5 mg/mL) were added to 100 μ L of antibodies (i.e. anti-cardiac troponin I IgG) for 1 hour to render thiol (-SH) functional group onto the antibody modality. After removing excess unreacted reagents by filtration through ZebaTM spin desalting columns, the purified thiolated-IgG molecules were mixed with 200 μ L of concentrated GNRs, followed by adding 100 μ L thiol-PEG (10 mg/mL) and incubation at room temperature overnight. The antibody-conjugated GNRs were separated by centrifugation at 8,000 rpm for 10 min and redispersed in 10 mM PBS to develop GNR bioprobes. Due to the immobilization of antibody onto the GNR surfaces, the surface plasmon resonance manifested by the nanorods is specifically responsive upon binding of the target antigen.

2.4 MNP mediated LSPR nanoarray biosensing

To construct a LSPR nanoarray biosensor, functional GNR probes were effectively immobilized onto a glass substrate in a chip-based format [19]. Briefly, the glass substrates were first treated with MPTMS solution (10% in ethanol) to enrich the surface with thiol groups. Then 10 μ L of functionalized GNR solution was dropped onto the designated spots in an array pattern on the substrates and incubated for 2 hrs. Afterwards, the substrate was washed three times with

DI water to remove weakly bound rods, resulting in a robust LSPR array chip. The absorption spectra of the GNRs in the nanoarray can be measured using a plate reader in a high-throughput fashion.

To perform a biosensing, a sample solution (10 μ L) with respective cTnI concentrations (2.5, 7.5, 15, and 30 ng/mL) was applied onto the nanosensing arrays and incubated 1 hr until reaction equilibrium. Afterwards, the UV-vis absorption spectra were measured to observe a red shift of the longitudinal plasmon peak which is proportional to the amount of cTnI binding. In the case of MNP mediated LSPR sensing, the sample solutions with various cTnI concentrations were first incubated with antibody-modified MNPs. The functional MNPs enabled cTnI extraction from the sample due to specific recognition to form MNP-captured cTnI complex. The nanoparticles were then retrieved by external magnet and washed with PBS buffer for purification. Afterwards, the resulting MNP-captured cTnI complex was rendered to the GNR nanoarray biochip to investigate the effect of sensitivity enhancement.

2.5 Instruments

TEM images of MNPs were taken on the JEOL-2010F transmission electron microscope. GNRs and MNPs on ITO-coated glass were characterized by Hitachi S5500 scanning transmission electron microscope (STEM). Absorption spectra were collected with a UV-vis spectrophotometer (Beckman Coulter) and Biotek plate reader. Zeta-potential measurements were performed on Zetasizer Nano ZS (Malvern Instruments, UK). Magnetic properties were measured by superconducting quantum interference device (SQUID) in Magnet and Low Temperature Facility at Northwestern University. Magnetization curve was recorded at room temperature with the magnetic field sweeping from -2 to 2 Tesla.

3. Results and discussion

3.1 Water dispersion of magnetic nanoparticles

The magnetic nanoparticles of varying sizes synthesized by thermal decomposition method were highly uniform in size and monodispersed in organic solvent due to the oleic acid capping agent (Fig. 1a-c). It was reported that oleic acid is chemisorbed on the particle surface via a carboxylic (-COOH) group while its nonpolar hydrocarbon chains expose outward [20]. The nonpolar branches stabilize the MNPs in organic solvents because they allow the creation of a surface that is mutually unreactive and repulsive, which is commonly considered to be steric stabilization. As shown in Figure 2a, the inward carboxylic groups were attached to iron oxide surface while the outward aliphatic hydrocarbon tails were suspended in the organic media. To efficiently transfer this magnetic nanostructure to an aqueous solution suitable for biological applications, sodium oleate (NaOL) was investigated as a phase transfer reagent. This is because NaOL possesses the same hydrocarbon chain structure as oleic acid, thereby allowing for a strong hydrophobic interaction. On the other end, the outer exposed carboxylate group of NaOL is hydrophilic to be water dispersible (Fig. 2a). The surface charge of NaOL-MNPs was -82.6 mV by zeta potential measurement, indicating that the hydrophilic portion (carboxylate) of the NaOL was indeed headed outwards to the aqueous media. The structure of oleate bilayer on the MNP surface is very similar to the surfactant bilayer on gold nanoparticles [21, 22]. The resulting MNPs were very stable in solution (Fig. S1), indicating a good NaOL protection of the magnetic core. Figure 1d-f shows the TEM images of MNPs (4, 8 and 15 nm) after phase transfer. There was little change in the water soluble nanostructure in terms of the monodispersity, morphology and particle size, as compared to the original nanoparticles in

organic solvent. The size distribution was uniform in aqueous solution (Fig. S2). The MNPs also exhibited excellent thermostability in aqueous solution for months. Fig. S3 shows the TEM images of the stable MNPs after 2-month storage at 4, 25 and 50 °C. These observations confirmed that NaOL was an effective phase transfer agent due to the high affinity of sodium oleate with the oleic acid on the MNP surface. The cytotoxicity of the aqueous magnetic nanoparticle was also evaluated to ensure its biocompatibility (Fig. S4).

3.2 Superparamagnetism before and after phase transfer to aqueous solution

The magnetic properties of the iron oxide nanoparticles (15 nm) before and after water dispersion were characterized using a superconducting quantum interference device magnetometer (SQUID). Strong paramagnetic behavior of all the Fe₃O₄ nanoparticle samples was confirmed in magnetic hysteresis measurements (Fig. 3). The insets of Figure 3(a-c) show the hysteresis loop on the smaller scale. The width of the hysteresis loop is an indication of the hardness of the magnetization; the wider loop implies a harder magnet. The data clearly showed that the NaOL-coated MNPs were the most magnetic in nature which was again confirmed by measuring the magnetic moment per nanoparticle. By fitting the magnetization curve with the Langevin function [23], the moment per particle was found to be 10,050 μ_B , 11,390 μ_B , and 14,740 μ_B respectively for oleic acid-coated (in solvent), CTAB-coated (aqueous) and NaOL-coated (aqueous) MNPs. For the CTAB-coated MNPs, the saturation magnetization (15 emu/g) was reduced after phase transfer, as compared to the MNPs in solvent (20 emu/g). The lower saturation magnetization of coated MNPs may be due to the presence of a dead magnetic layer on the surface [24, 25]. Indeed, the coercivities of the oleic acid-coated, CTAB-coated and NaOL-coated MNPs were estimated to be 7, 2 and 15 Oe respectively. These data confirmed the

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4 superiority of NaOL as the transfer agent. Figure 3d shows the temperature dependence zero
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6 field cooling (ZFC) of magnetization of the NaOL- MNPs measured with an applied magnetic
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8 field of 100 Oe from 380 to 5 K. It shows a maximum magnetization followed by a steady
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10 decrease up to 50K. At ca. 300K, the MNPs showed slight increment and thereafter decreased
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12 with the temperature drop. The maximum in the ZFC curve determines the blocking temperature
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14 (TB) where the thermal energy become comparable to the anisotropy energy barrier. For
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16 temperatures below TB, the nanoparticles magnetization aligns in the direction of the easy axis
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18 and cannot be changed due to the existence of the anisotropic energy barrier. The magnetization
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20 approaches low values due to the random orientation of the easy axes of the nanoparticles. As
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22 temperature increases an increasing number of nanoparticles acquire thermal energy comparable
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24 to the anisotropic energy that switch their magnetizations from the easy axes to the direction of
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26 the magnetic field which leads to an increase in the magnetization with temperature [26]. Fig. S5
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28 clearly demonstrates the superparamagnetism of the NaOL-MNPs in aqueous solution by an
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30 external magnet, indicating the retention of the magnetic property after phase transfer.
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41 *3.3 MNP mediated LSPR nanoarray biochip*

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43 To develop a specific biosensor, functionalization with biological receptors is necessary.
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45 NaOL coating not only facilitates the phase transfer from organic solvent to a biocompatible
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47 aqueous solution, but also benefits the functionalization by introducing carboxyl terminals. This
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49 surface modification results in customizable conjugation of fluorescence, oligonucleotides, or
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51 antibodies to the MNPs using the well-established EDC/NHS chemistry [11]. This strong
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53 covalent binding ensures the stability of the biofunctional moieties on the NaOL-MNP surfaces
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55 after immobilization during centrifuge and washing steps under optimal conditions. In this study,
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we specifically immobilized the anti-cTnI molecules onto the NaOL-MNPs to capture target cTnI in the sample. To confirm the successful biofunctionalization, FITC-labeled antibody was used and fluorescence microscopy showed the attachment of the antibody molecules on the MNPs. ELISA was also performed to confirm the bioactivity of the immobilized antibodies on the MNPs. There was little change in the binding affinity as compared to free form of antibody in buffer (data not shown).

To demonstrate the practical use of the water dispersed magnetic NPs after phase transfer, the MNPs were applied to a GNR biosensing chip to investigate the effect of enhancement on the assay sensitivity. Fig. 4 shows the schematic of the MNP mediated LSPR biosensing based on nanorod array. The biochip was fabricated by assembly of the GNRs onto the designated area in the glass substrate. Each dot was an independent nanosensor in an array format potential for a high-throughput biosensing. Biofunctionalization of the GNR sensor with anti-cTnI molecules enabled specific detection of cTnI, which is a clinical biomarker for myocardial infarction diagnosis [27]. When the cTnI-captured MNPs were applied to the sensor dot, the functional GNR would specifically bind to form a GNR-cTnI-MNP complex. Because the capture and detection anti-cTnI molecules are targeted for different epitopes on the cTnI antigen, the sandwich structure was constructed with minimal interference. This binding event caused the change in the refractive index of the medium surrounding the gold nanorods, which resulted in the longitudinal SPR shift.

Fig. 5 shows the absorption spectra of the GNR biochip in response to varying amount of cTnI sample without (a) and with (b) magnetic NP application. In a typical LSPR biosensor, biomolecule binding results in a red shift of the absorption peak because most biomolecules (i.e. DNA, antibody) have a higher refractive index ($n=1.4-1.6$) than water. At baseline, the LSPR

peak of the functional GNR sensor is 825 nm. Specific binding of cTnI shows a red shift of the longitudinal peak wavelength and the shift is proportional to the target analyte concentration in the sample [28]. As such, the LSPR peak gradually moved in the absorption spectra. The morphology of the waveform was little changed without peak broadening, indicating that the plasmon shift was not due to particle aggregation. The insets showed the calibration curve of the plasmonic shift as a function of the cTnI concentration from 2.5 to 30 ng/mL. The wavelength shift with the MNP application became more linear in the sensing range ($R^2 = 0.998$). It is interesting to compare the spectral response where MNP-captured cTnI sample showed a red shift magnitude twice of that in control without MNPs (Fig. 5c). For example, the LSPR shift was increased from 7 nm without MNPs to 17 nm with the MNP-mediated GNR detection of the cTnI at 15 ng/mL. This enhancement allows for a significantly improvement in the spectral sensitivity.

To further elucidate the mechanism of the MNP enhancement of the GNR biosensing, computational simulation was performed. Figure 6 shows the simulated surface plasmon intensity (SPI) of a gold nanorod in close proximity to a magnetic nanoparticle. It was found that the SPI is distance dependent between the nanorod and magnetic NP. When the placement of the MNPs is relatively far away (> 20 nm) from the GNR surface, the coupling effect between GNR and MNP is negligible and the SPI is comparable to the GNR without MNPs. However, the coupling effect is dramatically enhanced when the MNP is within 5 nm. In the COMSOL simulation, a strong SPI of the nanorod is observed which contributes to the enhancement of the spectral sensitivity in the cTnI detection. Due to the binding of the MNP captured cTnI with the anti-cTnI molecules on the GNR surface, the MNPs were within the effective distance, which explains the significant plasmonic shift as compared to the control sample. Additionally, as the

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4 dielectric constant of the MNP increases, the SPI of the nanorod increases. Since MNPs do not
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6 absorb visible light, its dielectric constant equals to the square of the refractive index [29, 30].
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8 Therefore, the high refractive index of MNP (~ 2.42) instead of the aqueous solution in control
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10 sample was attributed to the LSPR enhancement, which corroborates with previous reports [28,
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12 31]. All these data demonstrate the practical use of the phase transferred MNPs to enhance the
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14 LSPR biosensing due to the electromagnetic coupling effect.
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22 **Conclusions**

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25 We have demonstrated a facile phase transfer using NaOL to surface modify the iron oxide
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27 magnetic nanoparticles after thermal decomposition synthesis for two purposes: water dispersion
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29 and selective functionality. Compared to traditional strategies, this method is highly efficient,
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31 versatile, and biocompatible for biological applications. The aqueous MNPs with selected
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33 functional group facilitated the biofunctionalization with antibody for specific biosensing.
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35 Localized surface plasmon resonance based on gold nanorod biochip was found to be
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37 significantly enhanced by the functional MNPs after transfer. Additionally, the high refractive
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39 index of the MNP and electromagnetic coupling resulted in a 300% increase in the GNR biochip
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41 sensitivity for plasma protein detection. This study represents a new paradigm of the preparation
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43 of high quality aqueous MNPs for biomedical applications such as ultra-sensitive biosensing
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45 modality in a chip based format.
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Figure legend

Fig. 1. TEM images of magnetic nanoparticles of various sizes before (a, b, c) and after (d, e, f) phase transfer to aqueous solution. Insets: HRTEM images of monodisperse magnetic nanoparticles for respective size.

Fig. 2. Schematic of water dispersed MNP preparation and biofunctionalization. A: Phase transfer of oleic acid-coated MNP from organic solvent to aqueous using NaOL. B: Biofunctionalization of NaOL modified MNPs via EDC/NHS chemistry.

Fig. 3. Field dependence of magnetization measured at 300 K for (a) oleic acid-coated MNPs, (b) CTAB-coated MNPs, and (c) NaOL-coated MNPs. D: Temperature dependence of magnetization measured after zero-field cooling (ZFC) using 100 Oe for NaOL-coated MNPs.

Fig. 4. Magnetic nanoparticle enhanced LSPR biosensing based on gold nanorod biochip for specific detection of cTnI as a model.

Fig. 5. UV-vis absorption spectra of GNR biochip after binding with varying amount of cTnI antigen without (a) and with (b) functional MNPs. C: Enhancement of the longitudinal SPR shift of the GNR biochip by the application of MNPs in response to respective cTnI concentration.

Fig. 6. Computational simulation demonstrating the distance dependent enhancement of the plasmonic intensity by the MNPs.

(a)

(b)

Figure 1

Figure 2

(a)

(b)

Figure 3

Figure 4

(a)

(b)

Figure 5

Figure 6