Integrated magneto-plasmonic nanostructures-based immunoassay for galectin-3 detection†

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Cardiovascular diseases remain a leading cause of global mortality, highlighting the need for accurate diagnostic tools and the detection of specific cardiac biomarkers. Surface-enhanced Raman scattering (SERS) spectroscopy has proved to be a promising alternative diagnostic tool to detect relevant biomarkers compared to traditional methods. To our knowledge, SERS methodology has never been used to detect galectin-3 (Gal-3), a crucial biomarker for cardiovascular conditions. Our study aimed to develop plasmonic and magneto-plasmonic nanoplanforms for the sensitive immunodetection of Gal-3 using SERS. Spherical gold nanoparticles (AuNPs) were synthesized and functionalized with 11-mercaptoundecanoic acid (MUDA) to enable antibody binding and 4-mercaptobenzoic acid (4MBA) that served as a Raman reporter due to its intense Raman signal. Following bioconjugation with Gal-3 antibody, the AuNPs were employed in the immunodetection of Gal-3 in phosphate-buffer saline (PBS) solution, offering a limit of detection (LOD) of 12.2 ng mL\(^{-1}\) and a working range up to 120 ng mL\(^{-1}\). Furthermore, our SERS-based immunoassay demonstrated selectivity for Gal-3 (40 ng mL\(^{-1}\)) in the presence of other biomolecules such as α-amylase, bovine serum albumin and human C-reactive protein. As a proof of concept, we developed magneto-plasmonic nanoparticles composed of silica-coated magnetite decorated with the bioconjugated AuNPs aimed at enhancing the uptake and detection of Gal-3 via SERS coupled with Raman imaging. Our findings underscore the potential of SERS-based techniques for the sensitive and specific detection of biomarkers, holding significant implications for improved diagnosis and surveillance of cardiovascular diseases. Future research will focus on further optimizing these nanoplanforms and their transition into clinical settings.

Introduction

Cardiovascular diseases (CVDs) pose a significant challenge to global health, representing a major cause of mortality worldwide.1 According to data from 2019, approximately 17.9 million lives were lost to CVDs.1 The term “CVDs” encompasses a spectrum of pathological alterations affecting the heart and blood vessels, such as atherosclerosis, coronary heart disease, hypertension, peripheral arterial disease, among others.2

The rise in CVDs can predominantly be ascribed to prevalent lifestyle choices within populations.3,4 However, advancements in medical understanding, combined with increasingly sophisticated diagnostic techniques and proactive measures targeting CVD risk factors, have contributed to a decline in both mortality and morbidity rates.5,6

Early detection of CVDs through refined and precise methodologies, particularly by continuously monitoring inflammatory levels and identifying specific biomarkers, has notably decreased mortality rates.7,8 A diverse array of biomarkers is employed with the primary aim of forecasting specific cardiovascular risks, including myoglobins,7 cardiac troponins I and T,7 creatine phosphokinase,9 C-reactive protein,10 brain natriuretic peptide and N-terminal pro natriuretic peptide,11 creatine kinase and myocardial isoenzyme,13 and galectin-3.14

Galectin-3 (Gal-3) stands out as a unique member of the β-galactoside-binding lectin family, characterized by its chimaera-type structure and stable binding to non-immunological carbohydrates.14–16 Its influence extends across a spectrum of regulated cellular functions and biological processes.14–16 Clinically, Gal-3 serves as a biomarker not only in heart disease14–16 but also in viral infections,17,18 autoimmune diseases,19,20 diabetes,21 kidney disease22,23 and in tumour formations.24,25 In heart failure, blood Gal-3 levels (ranging from 1.4 to 94.8 ng mL\(^{-1}\)) mirror cardiac inflammatory responses, potentially as a marker for cardiac inflammation and fibrosis, depending on the underlying pathogenesis of heart failure.14–16 However, the

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precise mechanism driving elevated blood levels of Gal-3 remains incompletely elucidated.

Several methods have been proposed for detecting Gal-3, including radioactive immunoassay (RIA), enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), and electrochemical detection.\textsuperscript{26-31} While these methods offer sensitive detection of Gal-3, for example, the limit of detection (LOD) of commercially available ELISA kits is 0.29 ng mL\textsuperscript{−1},\textsuperscript{32} they come with inherent disadvantages. These include costliness, the requirement of two antibodies (in EIA, ELISA, and electrochemical approaches), generation of radioactive waste (in RIA), and time-intensive procedures due to their complexity, alongside the associated costs of equipment and specialized laboratories.\textsuperscript{33,34} Among these approaches, surface-enhanced Raman scattering (SERS) spectroscopy has garnered significant attention and undergone rigorous investigation for its prospective utility.\textsuperscript{35,36}

SERS spectroscopy is a sensitive vibrational technique that provides enhanced Raman signal of vestigial amounts of molecules upon their adsorption onto metallic nanostructures or colloidal plasmonic nanoparticles (NPs), such as gold and silver.\textsuperscript{37,38} While SERS holds significant advantages, its practical application faces challenges such as low reproducibility due to fluctuations in Raman signals and difficulty in selectively adsorbing molecular probes among potential interferences. To overcome these limitations, the label-SERS approach, also known as SERS tags, offers a powerful and versatile tool for the sensitive and selective identification of biomolecules of interest, including biomarkers.\textsuperscript{39-42} SERS-tags consist of nanoprobes based on metallic NPs decorated with Raman reporters and antibodies/aptamers, serving as markers for detecting target antigens in complex biological matrices.\textsuperscript{35,36,42} These Raman reporters are carefully selected based on their strong and characteristic Raman scattering signals, allowing for precise differentiation from background noise and offering specific insights into the presence and concentration of the target analyte.\textsuperscript{37,44}

The nanoparticles can be combined with magnetic particles, providing versatile bioconjugation, fast magnetic separation from complex solutions, and low toxicity.\textsuperscript{42,45} In a magnetic-plasmonic-based immunoassay, the repetitive washing steps can be quickly completed by applying an external magnetic field using a magnet, eliminating the need for laborious and time-consuming manual interventions such as centrifugations. Moreover, magnetic beads assist in concentrating the target molecules within samples, thereby enhancing the sensitivity of SERS-based immunoassays. For instance, Jun et al. have prepared magnetic-plasmonic NPs (Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}@Ag) for targeting specific cancer cells, such as breast cancer cells (SKBR3) and floating leukaemia cells (SP2/O).\textsuperscript{45} The targeted cancer cells can be easily separated from the untargeted cells using an external magnetic field, and their Raman signal is enhanced due to the presence of the plasmonic particles. Yap et al. have reported a novel microfluidic immunoassay chip using bifunctional plasmonic-magnetic micro/NPs (AuNPs anchored onto Fe\textsubscript{3}O\textsubscript{4} microspheres, followed by Ag coating), allowing for mixing, washing and SERS detection of rabbit t-IgG as low as 1 pg mL\textsuperscript{−1}.\textsuperscript{37} The immunoassay time could be reduced from 4 hours to 80 minutes, and the antibody binding specificity could be improved by ~70% compared to the corresponding non-microfluidic immunoassay. To the best of our knowledge, the SERS detection of Gal-3 using plasmonic or magneto-plasmonic probes has not been reported to date.

Here, we report sensitive and selective Gal-3 SERS detection using bioconjugated spherical gold nanoparticles (AuNPs) (Fig. 1). Gold nanospheres functionalized with 11-mercaptoundecanoic acid (MUDA) and 4-mercaptobenzoic acid (AuNPs@MUDA-4MBA) were bioconjugated with the Gal-3 antibody (IgG – anti-mG Gal-3, Ab) to detect Gal-3 at concentrations as low as 20 ng mL\textsuperscript{−1}. The selectivity tests demonstrate that Gal-3 can be detected with AuNPs@MUDA-4MBA-Ab even in the presence of potential interference species, namely \textalpha-amylase, bovine serum albumin (BSA), and human C-reactive protein (CRP).

As a proof-of-concept, magnetic beads (Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}) decorated with AuNPs@MUDA-4MBA were bioconjugated with Gal-3 Ab and used to detect Gal-3 using Raman imaging. In these SERS immunoassays, the magnetic phase is used to capture and extract Gal-3, and the plasmonic phase serves for its optical detection in buffer solution.

**Experimental**

**Materials**

The following chemicals were used as received: hydrogen tetrachloroaurate(III) trihydrate (HAuCl\textsubscript{4}·3H\textsubscript{2}O, 99.9%, Sigma-Aldrich), trisodium citrate dihydrate (Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}·2H\textsubscript{2}O, 99%, Sigma-Aldrich), potassium hydroxide (KOH, ≥85%, Sigma-Aldrich); potassium nitrate (KNO\textsubscript{3}, ≥98.0%, Sigma-Aldrich); ferrous(II) sulphate heptahydrate (FeSO\textsubscript{4}·7H\textsubscript{2}O, ≥97.0%, Sigma-Aldrich); tetracythyl orthosilicate [Si(OCH\textsubscript{3})\textsubscript{4}], TEOS, ≥99.0%, Sigma-Aldrich); ammonia (NH\textsubscript{4}OH, 25%, Sigma-Aldrich); (3-aminopropyl)triethoxysilane (H\textsubscript{2}N(CH\textsubscript{2})\textsubscript{3}-Si(OCH\textsubscript{3})\textsubscript{3}, APTES, ≥98.0%, Sigma-Aldrich); 11-mercaptoundecanoic acid (HS(CH\textsubscript{2})\textsubscript{10}COOH, MUDA, ≥96.0%, Sigma-Aldrich); sodium hydroxide (NaOH, ≥98.0%, Pronolab), 4-mercaptobenzoic acid (HSC\textsubscript{6}H\textsubscript{4}COOH, 4MBA, 99.0%, Sigma-Aldrich); ethanol (C\textsubscript{2}H\textsubscript{5}OH, ≥99.8%, Sigma-Aldrich); ethanoic acid (C\textsubscript{2}H\textsubscript{3}OH, ≥99.9%, Sigma-Aldrich); phosphate buffered saline (PBS); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (C\textsubscript{6}H\textsubscript{12}N\textsubscript{3}·HCl, EDC, ≥98%, Alfa Aesar); N-hydroxysuccinimide (C\textsubscript{2}H\textsubscript{5}NO\textsubscript{3}, NHS, 98%, Sigma-Aldrich); albumin from bovine serum (BSA, ≥98%, Sigma-Aldrich), \textalpha-amylase from porcine pancreas (≥10 units per mg, Sigma-Aldrich) C-reactive protein from human fluids (CRP, ≥90%, Sigma-Aldrich). Ultra-pure water was obtained through a Milli-Q system using a 0.22 μm filter (Synergy equipment, Millipore).
Functionalization of AuNPs and Fe₃O₄@SiO₂-APTES@Au composite with MUDA and 4-MBA

AuNPs were functionalized with MUDA and 4MBA following procedures adapted from the literature (Fig. 1(a and b)). Briefly, MUDA aqueous solution (1 mM) was prepared with ultrapure water and some drops of NaOH aqueous solution (0.01 M), to help in the powder dissolution. Then, colloidal AuNPs (10 mL) were mixed with 100 μL of MUDA (1 mM) and stirred at 700 rpm. After 15 minutes, an ethanolic solution of 4MBA (20 μL, 10 mM) and 10 μL of ammonia (15%) were added and stirred for 30 minutes. In the case of Fe₃O₄@SiO₂@AuNPs, 100 μL of the MUDA solution was added to 3 mL of the Fe₃O₄@SiO₂-APTES@AuNPs composite solution and left incubated in a mini rotor for 24 hours. Then, the Fe₃O₄@SiO₂-APTES@AuNPs@MUDA were magnetically separated from the supernatant, and 3 mL of ultrapure water was added. Subsequently, 20 μL of 4MBA (10 mM) and 10 μL of ammonia (15%) were added to the solution and incubated for 1 hour. After this period, the Fe₃O₄@SiO₂-APTES@AuNPs@MUDA-4MBA particles were magnetically separated and dispersed in 2 mL of ultrapure water.

Biofunctionalization of colloidal AuNPs and Fe₃O₄@SiO₂-APTES@Au composite with the Gal-3 antibody and BSA

The conjugation of AuNPs@MUDA-4MBA and Fe₃O₄@SiO₂-APTES@Au@MUDA-4MBA NPs with the Gal-3 antibody was
performed following the procedure described in our previous work (Fig. 1(a and b)). First, 1 mL of either AuNPs@MUDA-4MBA or Fe3O4@SiO2-APTES@AuNPs@MUDA-4MBA was mixed with 10 μL of EDC (5 mM) and 10 μL of NHS (7.5 mM), and the mixture was agitated in an orbital agitator for 30 minutes at 25 °C with a speed of 325 rpm. Then, the EDC/NHS activated AuNPs@MUDA-4MBA particles were centrifuged at 9500 rpm for 15 min and resuspended in the same volume of K2HPO4 (2.5 mM, pH 7.4). The activated Fe3O4@SiO2-APTES@Au@MUDA-4MBA particles were magnetically separated and redispersed in the K2HPO4 solution. Then, 25 μL of the Gal-3 antibody (Ab, 200 μg mL−1, PBS buffer) was added to the NPs and left to react for 60 minutes at 25 °C under orbital stirring (325 rpm). After that, 10 μL of 1% BSA (0.1 M PBS) were added to the NPs and let to react for another 10 minutes to block non-specific interactions. The resulting AuNPs@MUDA-4MBA@Ab or Fe3O4@SiO2-APTES@Au@MUDA-4MBA@Ab NPs were centrifuged at 6500 rpm for 11 minutes or magnetic separated, respectively. The supernatant was discarded, and the particles were resuspended in 800 μL of PBS (0.01 M, pH = 8).

**Detection of Gal-3 using SERS and Raman imaging**

In order to detect Gal-3 using either the AuNPs@MUDA-4MBA@Ab or Fe3O4@SiO2-APTES@Au@MUDA-4MBA@Ab, 30 μL of Gal-3 at different concentrations (0 ng mL−1, 20 ng mL−1, 40 ng mL−1, 80 ng mL−1 and 120 ng mL−1 for AuNPs and 0 ng mL−1, 10 ng mL−1, 40 ng mL−1 for Fe3O4@SiO2@Au) were added to 200 μL of nanoparticles suspension. The mixture was allowed to react for 60 minutes under orbital stirring (325 rpm) at 25 °C. Subsequently, the final colloidal solution was centrifuged or magnetically separated, respectively, and redispersed in PBS buffer solution (pH 7.4).

Samples of AuNPs@MUDA-4MBA@Ab and Fe3O4@SiO2-APTES@Au@MUDA-4MBA@Ab without and with Gal-3 were analyzed by SERS and Raman imaging (Fig. 1(2)). Samples with a volume of 20 μL were placed on a glass slide, and the solvent was evaporated at room temperature. Afterwards, the solid samples were analyzed by Raman spectroscopy using a 633 nm excitation line. The Raman spectra were acquired using 100 acquisitions per spectrum. A laser power at 0.1 mW with 2 s per acquisition with a typical diameter range observed for AuNPs synthesized via dynamic light scattering (DLS) for comparison, yielding an average size of 13.6 ± 0.1 nm. These values are in line with the typical diameter range observed for AuNPs synthesized via the citrate reduction method (approximately 15 nm), as reported in the literature.

As anticipated for spheroidal AuNPs with 14 nm of diameter, the synthesized colloidal AuNPs exhibit a distinctive ruby-red colouration and present a LSPR band in the optical spectrum at 521 nm (Fig. 2C). Additionally, the size of the AuNPs was

![Image](https://example.com/image-url)
assessed using UV-VIS spectroscopy, following the method described by Haiss et al.\textsuperscript{54} Eqn (1) accounts for the ratio between the absorbance of localized surface plasmon resonance band ($A_{\text{LSPR}}$) and the absorbance at 450 nm ($A_{450}$) in dependence of the logarithm of the particle diameter (particles between 5–80 nm). $B_1$ is the inverse of the slope ($m$) of the linear fit and $B_2 = B_0/m$ where $B_0$ is the intercept. We used the experimentally determined fit parameters for $B_1 = 3.00$ and $B_2 = 2.20$ because it results in an improved average deviation of ~11%. The calculated value for AuNPs diameter using eqn (1) was 16.6 nm.

$$d = \frac{A_{450}}{A_{\text{LSPR}}} B_2$$

The surface functionalization of AuNPs by citrate ions was confirmed through Fourier transform infrared spectroscopy (FTIR). Fig. 2D compares the FTIR spectrum of AuNPs alongside sodium citrate powder. Analysis of the AuNPs FTIR spectrum reveals the characteristic bands associated with citrate ions, including asymmetric stretching of COO$^-$ at 1582 cm$^{-1}$, symmetric stretching of COO$^-$ at 1390 cm$^{-1}$, the stretching vibrational mode of C–OH at 1281 cm$^{-1}$ and 1156 cm$^{-1}$, stretching of C–C and C–COOH at 908 cm$^{-1}$ and 842 cm$^{-1}$, respectively.\textsuperscript{55,56}

To enable affinity towards a specific analyte, it is imperative to bioconjugate the surface of AuNPs with antibodies, thereby enhancing the selectivity and sensitivity of the assay. To achieve this, the AuNPs’ surface was initially modified using MUDA (AuNPs@MUDA), a long-chain thiol compound featuring a carboxyl-terminal group (–COOH). The thiol terminal group facilitates the exchange with the citrate ions on the AuNPs’ surface due to sulfur’s higher affinity for gold, resulting in the formation of a robust covalent bond S–Au. Simultaneously, the –COOH group forms a covalent bond with the antibodies via amine groups. Subsequently, the AuNPs@MUDA were further modified with 4MBA (AuNPs@MUDA-4MBA), serving as a Raman reporter to monitor variations in the SERS intensity corresponding to changes in Gal-3 concentration within the sample. Characterization of the resultant AuNPs involved employing diverse techniques to confirm the presence of thiol compounds (MUDA and 4MBA) on the AuNPs’ surface while ensuring the particles retained their optical properties and maintained their spherical morphology.

The TEM image of AuNPs@MUDA-4MBA, depicted in Fig. 3A, shows spheroidal particles with an average diameter of 15.6 ± 3.9 nm. This observation indicates that the surface modification of the AuNPs does not compromise their spheroidal shape. Analysis of the UV-VIS spectrum of AuNPs@MUDA-4MBA reveals a subtle deviation of the LSPR band towards higher wavelengths ($\Delta = 5$ nm) compared to the AuNPs (Fig. 3B). This redshift in the AuNPs’ LSPR can be attributed to changes in the dielectric environment, suggesting that the citrate ions were indeed replaced by MUDA and 4MBA molecules, which are now attached to the AuNPs’ surface.

FTIR spectroscopy was used to confirm the presence of MUDA on the surface of AuNPs. Fig. 3C shows the FTIR spectra of AuNPs@MUDA-4MBA, MUDA and 4MBA powders for comparison. While the vibrational bands of citrate ions at 1389 cm$^{-1}$ and 1569 cm$^{-1}$, attributed to C–O–H stretching vibration mode and symmetric C=O stretching vibration, respectively, are still observed, the appearance of bands at 2916 cm$^{-1}$ and 2848 cm$^{-1}$, indicative of the C–H stretching vibrations, confirmed the presence of MUDA molecules at the surface of the Au particles.\textsuperscript{48,57} Additionally, FTIR analysis of AuNPs@MUDA (without 4MBA) yielded a similar spectrum, further supporting that the observed bands at 2916 cm$^{-1}$ and 2848 cm$^{-1}$ are from the MUDA molecules (data not shown).

Subsequently, Raman spectroscopy was employed to validate the presence of 4MBA molecules on the AuNPs’ surface (Fig. 3D). Colloidal solutions of AuNPs and AuNPs@4-MBA (without MUDA) were also analyzed for comparative purposes. As anticipated, the Raman spectrum of AuNPs exhibited no discernible Raman bands under these experimental conditions. In contrast, both Raman spectra of AuNPs@MUDA-4MBA and AuNPs@4MBA showcased characteristic Raman bands of 4MBA, including the band at 1078 cm$^{-1}$ assigned to the aromatic ring breathing, symmetric C–H in-plane bending and C–S stretching, and the band at 1586 cm$^{-1}$ attributed to the ring C–C stretch and asymmetric C–H in-plane bending.\textsuperscript{58,59} Notably, there is a slight decrease in the Raman signal of the 4MBA
molecules on the AuNPs@MUDA-4MBA particles compared to the Raman signal of 4MBA adsorbed on the AuNPs alone (AuNPs@4MBA). In the latter case, the 4MBA is adsorbed on the AuNPs’ surface, which is fully available. However, in the AuNPs@MUDA-4MBA particles, the MUDA molecules are already on the AuNPs’ surface. Thus, both organic molecules (MUDA and 4MBA) will compete for the AuNPs’ surface. This competition likely explains the slight decrease of the Raman signal of 4MBA for the AuNPs@MUDA-4MBA particles observed in Fig. 3D(b and c). All the SERS studies on Gal-3 were performed using AuNPs@MUDA-4MBA particles.

Furthermore, the AuNPs@MUDA-4MBA particles were immobilized on a glass slide and subjected to Raman imaging to investigate the feasibility of tracking the distribution of the Raman reporter on the AuNPs using this technique. Fig. 3E and F depict the optical and corresponding 2D Raman images of 4MBA adsorbed on the AuNPs’ surface. Raman imaging was conducted over a surface area of 30 μm × 30 μm by accumulating a full Raman spectrum at each pixel (22,500 Raman spectra) and integrating the Raman band of 4MBA at 1078 cm⁻¹. Stronger SERS signal intensities from 4MBA imply higher amounts of the molecular probe, depicted by a brighter color in Fig. 3F. Collectively, these findings validate the successful surface modification of AuNPs with MUDA and 4MBA molecules.

**Detection of Gal-3**

The conjugation of AuNPs with Gal-3 antibody (Ab) involved using EDC and NHS molecules to form an amide bond between –COOH groups at AuNPs surface and Ab –NH₂ groups.⁶⁴,⁶⁰ Although the aim is to bind Ab to MUDA molecules, it is worth considering the possibility that Ab can bind to 4MBA molecules, which also have a carboxylate group (–COO⁻) at one of their ends with a shorter carbon chain.⁶¹ Following antibody binding, the gold surface undergoes further modification with BSA to prevent non-specific interactions.⁶² Post-antibody conjugation, the AuNPs underwent SERS analysis to elucidate whether the addition of antibodies influenced the Raman signal of 4MBA. The obtained results, depicted in Fig. 4A(a and b), reveal no significant difference in the Raman signal intensity of 4MBA post-conjugation with the antibody. However, upon closer inspection of the Raman spectrum in Fig. 4A(b), discernible alterations were noted, including an increase in the band at 1420 cm⁻¹ (–COO⁻) and the disappearance of the band at 1690 cm⁻¹ (C=O). These observed changes in the Raman spectrum of 4MBA for the AuNPs@MUDA-4MBA@Ab sample can be attributed to variations in the pH of the medium. The bioconjugation of AuNPs with antibodies occurs in an alkaline environment (pH 8), where the carbonyl group of 4MBA exists predominantly in its deprotonated form.⁶¹ This result also confirms that the antibodies are predominantly bioconjugated with MUDA molecules instead of 4MBA because the 4MBA molecules have their carbonyl group free to be deprotonated.

The sensitivity and selectivity of detecting Gal-3 using the proposed SERS-based AuNPs were evaluated. The interference of the buffer solution on the 4MBA SERS detection was first assessed. The SERS spectrum obtained for AuNPs@MUDA-4MBA@Ab dispersed in a PBS buffer solution (pH 7.4) without Gal-3 (0 ng mL⁻¹, control sample) is shown in Fig. 4A(c). A decrease in the intensity of the Raman bands for 4MBA is observed, likely due to changes in the pH of the buffer solution; however, the 4MBA Raman signal remains stable. Thus, the experiment for plotting the intensity of the 1078 cm⁻¹ 4MBA Raman band for various Gal-3 concentrations was carried out in PBS.
The SERS spectra of 4MBA corresponding to different concentrations of Gal-3 are depicted in Fig. 4B (inset image). As the concentration of Gal-3 increases from 20 ng mL\(^{-1}\) to 120 ng mL\(^{-1}\), the intensity of the SERS band of 4MBA also increases. The SERS intensities of 15 spectra at different points on the sample were averaged to obtain a reproducible signal. The tests were conducted for two distinct AuNPs@MUDA-4MBA batches, and the standard deviation was calculated. The tests were conducted for two distinct AuNPs@MUDA-4MBA batches, and the standard deviation was calculated. The plot of the intensity of the 1078 cm\(^{-1}\) band as a function of Gal-3 concentration is presented in Fig. 4B, revealing a strong linear relationship with \(R^2\) of 0.9893. The limit of detection (LOD), calculated using eqn S1 (ESI),† was found to be 12.2 ng mL\(^{-1}\).

The increase of the 4MBA Raman signal intensity with Gal-3 concentration suggests that the bioconjugated AuNPs aggregate more in the presence of higher amounts of Gal-3 and is in line with other works.\(^{64,65}\)

To gather more evidence on AuNPs aggregation, the response of AuNPs-based immunoassays to varying concentrations of Gal-3 was analyzed using UV/VIS spectroscopy (Fig. S2†). When the Gal-3 concentration increased, we observed a slight redshift (\(\Delta = 3\) nm) and a decrease in the absorbance of the LSPR band of the AuNPs at 529 nm. The AuNPs aggregation through the interaction of Gal-3 with antibody-bioconjugated Au nanoprobes has been reported by M. António et al.\(^{48}\) In this work, the authors have observed a decrease in the LSPR absorption band of AuNPs with the increase of the Gal-3 concentration, associating this observation to the formation of homodimers and, consequently, the aggregation of the AuNPs. It has been reported in the literature that Gal-3 can form homodimers in the absence of carbohydrate-binding ligands.\(^{66}\)

The formation of homodimers can induce interparticle linking by attaching to two AuNPs@MUDA-4MBA@Ab, leading to the formation of aggregates at high Gal-3 concentration, therefore enhancing the Raman signal of the Raman reporter.

The selectivity of the SERS AuNPs@MUDA-4MBA@Ab probes to detect Gal-3 (40 ng mL\(^{-1}\)) in the presence of potential interfering species in identical concentration, namely \(\alpha\)-amylose, BSA, and CRP, was thoroughly evaluated. As displayed in Fig. 4C, the intensity of the Raman band for 4MBA adsorbed on the samples in the presence of Gal-3 at a concentration of 40 ng mL\(^{-1}\) in PBS buffer solution (\(I_{1078\text{cm}^{-1}}\): 495 ± 86) and mixed with \(\alpha\)-amylose (\(I_{1078\text{cm}^{-1}}\): 516 ± 135), BSA (\(I_{1078\text{cm}^{-1}}\): 473 ± 109) and CRP (\(I_{1078\text{cm}^{-1}}\): 471 ± 70). The obtained values are very close to each other, demonstrating that this immunoassay system exhibits high selectivity for Gal-3 and can effectively detect Gal-3 by SERS.

**Magneto-plasmonic probes for Gal-3 SERS detection**

Motivated by our promising findings, we attempted to integrate plasmonic probes with magnetite NPs. Our objective was to explore the potential of the resultant magneto-plasmonic probes in the efficient collection and magnetic preconcentration of Gal-3, while simultaneously enabling its detection through SERS coupled with Raman imaging. Initially, magnetite NPs (Fe\(_3\)O\(_4\)) were synthesized via the oxidative hydrolysis of iron sulfate heptahydrate(u) and subsequently coated with a silica layer by catalyzing the hydrolysis and condensation of TEOS in an alkaline medium (the experimental procedure is described in the ESI).\(^{67,68}\) This SiO\(_2\) shell protects the magnetic nanoparticles from oxidation and provides silanol groups (Si–OH) on their surface for further functionalization.

TEM analysis (Fig. 5A) shows spheroidal Fe\(_3\)O\(_4\) particles with an average particle size of 81 ± 21 nm. The silica coating layer measures approximately 18 ± 3.5 nm thickness (Fig. 5B), resulting in a total size of the Fe\(_3\)O\(_4\)@SiO\(_2\) NPs of 117 ± 31 nm. The powder X-ray diffractogram of magnetite (Fig. S3A†) reveals distinct diffraction features at 19.5°, 30°, 35.5°, 43°, 54°, 57.5° and 63° that correspond to the (111), (220), (331), (400), (422),
(511) and (440) planes, respectively, of the inverse spinel structure of magnetite [JCPDS file no. 19-0629] (International Centre for Diffraction Data-Powder Diffraction File [ICDDPDF] No. 00-005-0566).

Then, the Fe3O4@SiO2 particles underwent functionalization with APTES, a silane agent containing terminal amine groups. These amine groups serve to impart positive charges to the surface of the Fe3O4@SiO2 particles, thereby facilitating the coupling of the AuNPs (experimental procedure is described in the ESI†).

Zeta potential and elemental microanalysis results of Fe3O1, Fe3O4@SiO2 and Fe3O4@SiO2-APTES NPs are provided in Table S1.† Zeta potential values reveal negative surface charges for both Fe3O1 and Fe3O4@SiO2 NPs, with values of −35.0 mV and −45.5 mV at pH = 5, respectively. The negative surface of Fe3O4@SiO2 is attributed to the presence of silanol groups (Si–OH) on the NPs surface and indicates high colloidal stability within the tested pH range (pH = 5). Confirmation of APTES functionalization was demonstrated by the inversion in surface charge to a positive value (+19.5 mV) for Fe3O4@SiO2-APTES NPs, along with the detection of nitrogen in the elemental analysis. This positive surface charge arises from the protonation of amine groups on the NP surface at pH = 5.

The as-prepared colloidal citrate-capped AuNPs were attached to the surface of Fe3O4@SiO2-APTES through electrostatic interactions. The negatively charged AuNPs (−41.2 mV) interacted with the positively charged amine groups of the APTES molecules present on the silica shell. TEM images shown in Fig. 5C provide visual confirmation of the successful decoration of Fe3O4@SiO2-APTES NPs with AuNPs, yielding Fe3O4@SiO2@Au NPs.

Subsequently, the decorated particles were functionalized with MUDA and 4MBA. The optimization of the MUDA functionalization process on the Fe3O4@SiO2-APTES@Au particles involved varying the functionalization time (1 h, 24 h) and subsequent FTIR analysis (Fig. S3†). The FTIR analysis revealed that the 1 hour functionalization process did not exhibit signals indicative of MUDA presence on the particles, likely due to the short reaction time, resulting in limited attachment of MUDA to the AuNPs. However, the 24 hours functionalization yielded distinct bands at 2847 cm−1 and 2910 cm−1 corresponding to the C–H bond vibrations, confirming the successful presence of MUDA on the particles.†

Then, the resulting NPs were functionalized with 4MBA by dispersing them in an ethanolic 4MBA solution for 1 h.

The Raman spectrum of the resulting Fe3O4@SiO2-APTES@Au@MUDA-4MBA NPs (depicted in Fig. 6A), exhibited the characteristic features of 4MBA.58,59 Raman mapping (Fig. 6B) was conducted to determine the distribution of 4MBA on the NPs monitoring the intensity of the band at 1078 cm−1. The 4MBA molecules were observed predominantly at the tips of the aggregates (brighter areas), corresponding to the regions with higher SERS intensity. Since we know that 4MBA will covalently bind to the AuNPs, this observation not only elucidates the distribution of 4MBA molecules but also suggests the distribution of AuNPs, indicating their higher concentration at the tips of the magnetic particle aggregates, consistent with the microscopy image presented in Fig. 5C.

To detect Gal-3, the Fe3O4@SiO2-APTES@Au@MUDA-4MBA NPs were conjugated with the corresponding antibody and the Raman spectrum of the resulting particles (Fe3O4@SiO2-APTES@Au@MUDA-4MBA@Ab) is presented in Fig. S4b.† Consistent with our observations for the AuNPs (Fig. 4A), the SERS spectrum of 4MBA is intensified upon the bioconjugation of the NPs with the antibody.

In a proof-of-concept, the final magnetic NPs were tested in detecting Gal-3 at various concentrations (0, 10, and 40 ng mL−1) in PBS. Following exposure to the Gal-3 solution, the particles underwent magnetic separation and subsequent analysis using SERS coupled with Raman imaging. The Raman signal of 4MBA in the NPs remained unaffected by the presence of PBS buffer (control sample, in the absence of Gal-3), exhibiting identical intensity to that observed when measured in ultrapure water (Fig. S3b and c†). An increase in bright spots is observed in the Raman images presented in Fig. 7, correlating with increasing Gal-3 concentration (0–40 ng mL−1). This result indicates that as the Gal-3 concentration rises, the 4MBA Raman intensity also increases, a trend confirmed by the Raman spectra illustrated in Fig. 7D. This finding aligns with the results obtained for the AuNPs, wherein the AuNPs tend to aggregate in the presence of higher Gal-3 concentrations due to the formation of homodimers.48 In this case, the Gal-3 molecules form homodimers and will interact with two AuNPs at the surface of Fe3O4@SiO2 beads, inducing AuNP aggregation and consequently enhancing the Raman signal of 4MBA. It is worth noting that this analytical approach employing Raman imaging is relatively underexplored for immunoassay detection, with...
only a limited number of studies demonstrating its application in this specific context.^{69–72}

Conclusions

In summary, we have developed plasmonic nanoplatforms based on AuNPs@MUDA-4MBA@Ab for the sensitive detection and quantification of Gal-3 employing SERS spectroscopy. This is the first reported method for SERS-based detection of Gal-3. Our study demonstrated effective immunodetection of Gal-3 in PBS at physiological pH (7.4) across a broader working range (12.2–120 ng mL\(^{-1}\)) and with a faster turnaround time compared to traditional techniques such as ELISA. Additionally, these AuNPs-based immunoassays can be miniaturized, paving the way for the development of portable diagnostic devices that are suitable for point of care testing. We observed a proportional increase in the 4MBA Raman signal concomitant with increasing Gal-3 concentration, attributable to the formation of Gal-3 homodimers triggering AuNP aggregation. Additionally, the development of magneto-plasmonic nanoparticles (Fe\(_3\)O\(_4\)@SiO\(_2\)-APTES@Au@MUDA-4MBA) provides a promising avenue for Gal-3 detection via SERS coupled with Raman imaging. The use of magnetic separation in the immunoassays significantly reduces the detection time (~1 h 20 min) compared to commercially available ELISA kit (~2 h 30 min).\(^{32}\) Overall, our findings highlight the promise of SERS-based techniques in improving the diagnosis and surveillance of cardiovascular diseases. Future investigations should prioritize the optimization of these nanoplatforms and validate the applicability of our approach on a larger scale and in biofluids such as human plasma or saliva.

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions


Conflicts of interest

There are no conflicts to declare.

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Notes and references


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