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Synthesis and characterisation of PNA conjugated chitosan coated magnetic iron oxide nanoparticles

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Abstract

The present study was undertaken to synthesize and characterize of PNA conjugated chitosan coated magnetic iron oxide nanoparticles for the nanopurification of Malabari buck semen. Iron oxide nanoparticles (IONPs) have attracted increasing interest in animal reproductive biotechnology due to their magnetic properties, biocompatibility and amenability to surface functionalisation. The present study aimed to synthesise magnetite iron oxide nanoparticles (Fe_3O_4), functionalise them with chitosan, and bioconjugate peanut agglutinin (PNA) lectin to develop a biologically relevant nanoparticle system for buck semen nanopurification. Magnetite nanoparticles were synthesised using the chemical co-precipitation method and surface modified with chitosan to improve colloidal stability and provide reactive functional groups. Lectin conjugation was achieved through glutaraldehyde-mediated covalent crosslinking. The synthesised nanoparticles were characterised using UV-visible spectroscopy, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR) and zeta potential analysis. UV-visible spectroscopy confirmed nanoparticle formation and preservation of the iron oxide core following surface modification. SEM revealed quasi-spherical to irregular nanoparticles within the nanoscale range. FTIR spectra confirmed successful chitosan coating and PNA immobilisation through characteristic functional group signatures. Zeta potential analysis indicated moderate colloidal stability with a predominantly negative surface charge after lectin conjugation. These findings demonstrate the successful synthesis, functionalisation and bioconjugation of IONPs, supporting their suitability for application in semen nanopurification strategies.

Keywords: Iron oxide nanoparticles, chitosan, PNA lectin conjugation, UV-visible spectroscopy, FTIR, SEM

Introduction

Nanotechnology has emerged as a rapidly advancing interdisciplinary field with transformative potential in biomedical and veterinary sciences. The unique physicochemical properties of nanoparticles, including their nanoscale dimensions, high surface area-to-volume ratio, adaptable surface chemistry and enhanced interaction with biological systems, have driven their widespread application in diagnostics, therapeutics and imaging. In veterinary medicine, these attributes have facilitated the development of novel strategies in disease diagnosis, targeted drug delivery and reproductive biotechnology (Rodrigues *et al.*, 2023; Meng *et al.*, 2024) [12, 6].

Among the various nanomaterials explored to date, iron oxide nanoparticles (IONPs) have attracted substantial interest due to their superparamagnetic behaviour, chemical stability and favourable biocompatibility. Their ability to respond to external magnetic fields while remaining well dispersed in biological environments renders IONPs particularly suitable for applications such as magnetic bioseparation, targeted drug delivery and imaging enhancement (Stolnik *et al.*, 1995; Meng *et al.*, 2023) [13, 7]. In the context of veterinary medicine, these distinctive properties have expanded the potential use of IONPs in advanced reproductive interventions, thereby positioning them as promising tools for improving the efficiency of assisted reproductive technologies.

Magnetite (Fe_3O_4) nanoparticles synthesised using chemical co-precipitation methods are

widely preferred owing to the simplicity, cost-effectiveness, scalability and reasonable control over particle size offered by this approach (Ba-Abbad *et al.*, 2022) ^[1]. Despite these advantages, bare iron oxide nanoparticles are prone to aggregation as a result of strong magnetic dipole-dipole interactions and are susceptible to surface oxidation under aqueous and physiological conditions. These inherent limitations compromise their colloidal stability and biological performance. To address these challenges, surface modification using stabilising agents and biopolymers such as silica, dextran and chitosan has been extensively employed to improve nanoparticle stability, prevent aggregation and enhance functional versatility. (Deng *et al.*, 2005; Yousef *et al.*, 2020; Panigrahi *et al.*, 2023) ^[2, 14, 10].

Chitosan, a naturally derived polysaccharide, has gained particular prominence as a coating material for IONPs owing to its biodegradability, biocompatibility, low toxicity and inherent cationic nature. Coating IONPs with chitosan provides both electrostatic and steric stabilisation, enhances aqueous dispersibility, and introduces reactive amino and hydroxyl functional groups on the nanoparticle surface. These functional moieties facilitate subsequent bioconjugation with a wide range of biomolecules (Kumar and Dixit, 2017; Panigrahi *et al.*, 2023) ^[5, 10]. Collectively, these attributes enhance the suitability of chitosan-coated IONPs for diverse biological applications, including advanced reproductive biotechnologies.

Lectins are carbohydrate-binding proteins that exhibit high specificity towards glycoproteins and glycolipids present on cell surfaces. In reproductive biology, lectins have been widely utilised for the identification and separation of functionally distinct cell populations based on surface carbohydrate expression patterns (Gregorio-Jauregui *et al.*, 2014) ^[3]. Peanut agglutinin (PNA) lectin, in particular, has been extensively used owing to its affinity for galactose-containing residues, which are differentially expressed on spermatozoa depending on membrane integrity and functional status. The conjugation of lectins onto magnetic nanoparticle surfaces combines the magnetic responsiveness of IONPs with the biological specificity of lectins, enabling the development of targeted and efficient biofunctional nanocomplexes for cell separation applications (Yousef *et al.*, 2020) ^[14].

Despite increasing interest in lectin-functionalised magnetic nanoparticles, systematic studies addressing their synthesis, surface modification and detailed physicochemical characterisation for veterinary and animal husbandry applications remain limited. Comprehensive characterisation is essential to confirm nanoparticle stability, surface functionalisation and biological suitability prior to application. Accordingly, the present study aimed to synthesise magnetite IONPs, functionalise them with chitosan and bioconjugate peanut agglutinin lectin, followed by physicochemical characterisation using UV-visible spectroscopy, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR) and zeta potential analysis. The findings are expected to provide foundational insights for the development of lectin-functionalised Fe₃O₄ nanoparticles for semen nanopurification and related reproductive biotechnological applications in veterinary science.

Materials and Methods

Synthesis of iron oxide nanoparticles

Magnetite IONPs were synthesised using a co-precipitation technique following the method described by Ba-Abbad *et al.* (2022) ^[1] with minor modifications. Ferrous sulphate heptahydrate (FeSO₄·7H₂O) and ferric chloride hexahydrate (FeCl₃·6H₂O) were dissolved separately in distilled water to obtain a molar ratio of 2:1. Each salt was dissolved in 100 mL of distilled water under continuous magnetic stirring at room temperature. After complete dissolution, the two solutions were combined in a 500 mL beaker and heated to 40 °C. A 10% ammonium hydroxide (NH₄OH) solution was added dropwise to the heated mixture under vigorous stirring at 500 rpm until the pH reached 11.0. The reaction mixture was subsequently maintained at 65 °C with continuous stirring for two hours to ensure complete nanoparticle formation. The suspension was then allowed to cool to room temperature. The precipitate obtained was washed repeatedly with ultrapure water (Milli-Q®, Millipore, USA) to remove unreacted reagents until neutral pH was achieved. A final wash with absolute ethanol was performed, and the nanoparticles (Fig. 1) were dried in a hot air oven at 75 °C for 24 h.

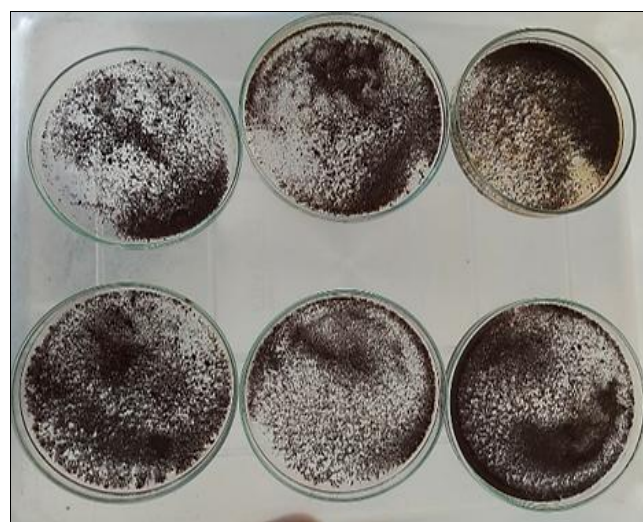


Fig 1: Fe₃O₄ nanoparticles synthesised by co-precipitation technique

Functionalisation of iron oxide nanoparticles with chitosan

Functionalisation of the synthesised IONPs was performed using chitosan as a surface coating agent according to the procedure described by Yousef *et al.* (2020) ^[14] with slight modifications. Briefly, 0.25 g of chitosan was dissolved in 50 mL of 1% (v/v) acetic acid solution under continuous stirring to obtain a homogeneous solution. To this, 2 g of dried magnetite nanoparticles were added and the mixture was stirred continuously for 30 min to ensure uniform dispersion of the nanoparticles.

Subsequently, 50 mL of 1 M sodium hydroxide (NaOH) solution was added dropwise to the dispersion to induce precipitation of chitosan onto the nanoparticle surface. The resulting chitosan-coated IONPs were collected and washed repeatedly with deionised water until a neutral pH was achieved. The functionalised nanoparticles were then dried in a hot air oven at 80 °C and stored for further use (Fig. 2).



Fig 2: Chitosan coated nanoparticles

Bioconjugation of Peanut Agglutinin (PNA) lectin onto chitosan-coated IONPs

Bioconjugation of PNA lectin onto chitosan-coated IONPs was carried out according to the method described by Gregorio-Jauregui *et al.* (2014) [3], with minor modifications. Briefly, 1 g of chitosan-functionalised IONPs was suspended in 10 mL of 25% glutaraldehyde solution and incubated at 37 °C for 4 h to activate the nanoparticle surface.

The activated nanoparticles were subsequently washed thoroughly with ultrapure water (Milli-Q®, Millipore, USA) to remove unreacted glutaraldehyde. Thereafter, the nanoparticles were incubated with 5 mL of PNA lectin solution (0.04 mg/mL) at 4 °C for 12 h under gentle agitation to facilitate covalent immobilisation of the lectin. The PNA-conjugated IONPs were separated using an external magnetic field and washed with phosphate-buffered saline (PBS). The final nanoparticle preparation was resuspended in PBS and stored at 4 °C until further use.

Characterisation of iron oxide nanoparticles

The synthesised IONPs were characterised at different stages, including bare nanoparticles, chitosan-functionalised nanoparticles, and PNA-conjugated nanoparticles. Characterisation was performed to assess particle size, morphology, surface charge, and chemical composition using UV-visible spectroscopy, FTIR, SEM, and zeta potential analysis.

UV-visible spectroscopy

UV-visible spectroscopic analysis was carried out using a BIOMATE 3S UV-Visible spectrophotometer (Thermo Fisher Scientific, USA). Triple-distilled water was used as a blank, and all measurements were performed using quartz cuvettes. Absorption spectra were recorded over a wavelength range of 200-500 nm at room temperature.

Size and morphological analysis

The size and morphology of the IONPs were analysed using a TESCAN VEGA3 scanning electron microscope (Czech

Republic). The instrument operates with a tungsten heated cathode and an accelerating voltage ranging from 200 eV to 30 keV. Samples were mounted on a motorised five-axis stage and examined under high and low vacuum conditions using secondary electron and backscattered electron detectors. Image acquisition and analysis were performed using TESCAN proprietary software.

Chemical structure analysis

Fourier-transform infrared (FTIR) spectroscopy was performed using a JASCO FT/IR-6600 spectrometer (Japan) equipped with an attenuated total reflectance (ATR) accessory. Spectra were recorded over the range of 400-4,000 cm^{-1} with a resolution of 0.4 cm^{-1} . All measurements were conducted in automatic scan mode, and spectral data were processed using JASCO Spectra Manager software.

Surface charge analysis

Zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS (United Kingdom). The instrument employs M3-PALS technology, which determines particle mobility through laser Doppler electrophoresis under an applied electric field. Measurements were performed using a minimum sample volume of 20 μL , with temperature controlled within the range of 0-90 °C. Data acquisition and analysis were performed using Malvern software to ensure accuracy and reproducibility.

Results and Discussion

UV-Visible spectroscopy

UV-visible spectroscopy is widely used as a rapid and reliable technique for the preliminary characterisation of nanoparticles, as it provides information regarding nanoparticle formation and optical stability (Zhang *et al.*, 2016) [15]. In the present study, bare IONPs exhibited a distinct absorption band in the range of 280-285 nm along with a broad absorption tail extending into the visible region. This spectral pattern is characteristic of nanoscale iron oxide and confirmed the successful synthesis of IONPs.

Following surface modification, chitosan-coated IONPs retained the characteristic absorption band at 280-285 nm, indicating preservation of the iron oxide core after coating. The slight broadening and reduction in absorption intensity observed in the chitosan-coated nanoparticles may be attributed to alterations in the local dielectric environment surrounding the nanoparticle surface due to the polymer coating. Similar spectral features have been reported for chitosan-coated magnetite nanoparticles, which typically exhibit absorption in the 280-310 nm range (Yousef *et al.*, 2020) [14]. Minor absorption features detected in the 250-270 nm region are likely associated with electronic transitions related to chitosan, further supporting successful surface functionalisation. Overall, the UV-visible spectral characteristics confirmed effective nanoparticle synthesis and surface modification.

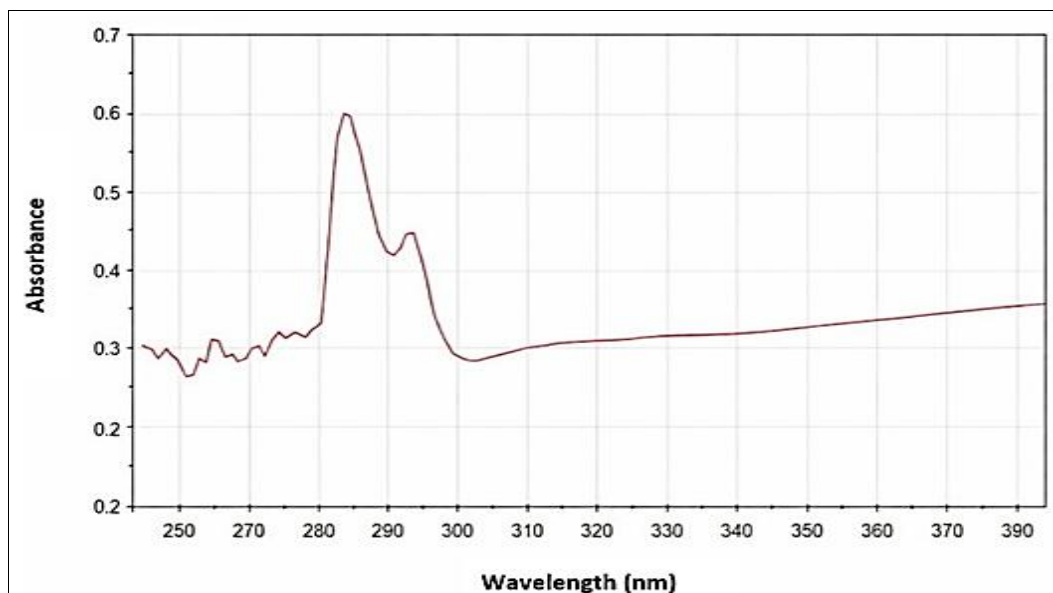


Fig 3: UV-visible spectroscopy- bare iron oxide nanoparticles

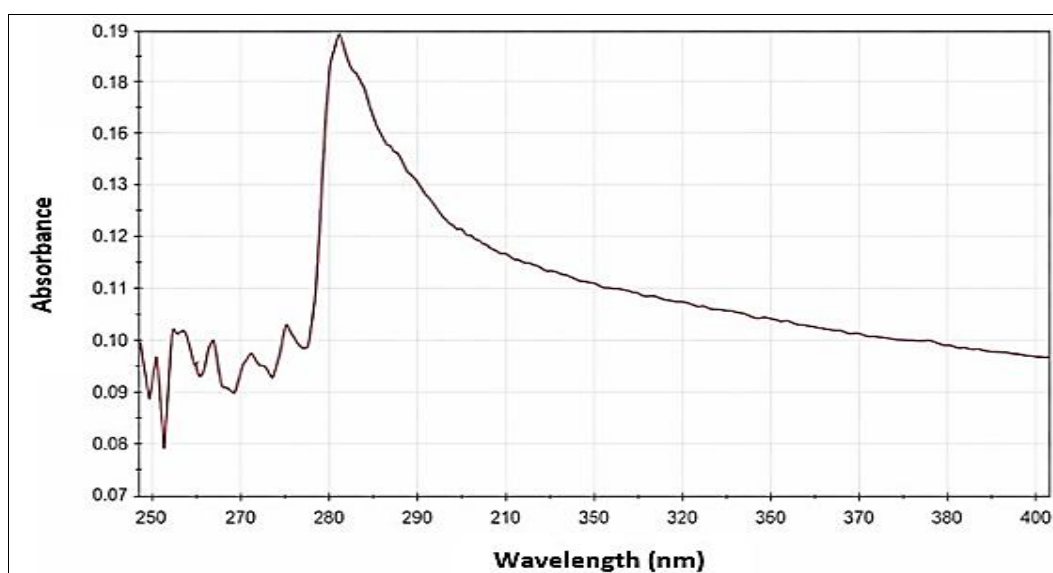


Fig 4: UV-visible spectroscopy- chitosan-coated iron oxide nanoparticles

Nanoparticle size and morphology

Particle size and morphology are critical parameters influencing the stability and biological performance of nanoparticle systems (Mohanraj and Chen, 2006) [8]. In the present study, SEM analysis revealed that chitosan-coated

iron oxide nanoparticles were predominantly quasi-spherical to irregular in shape, with particle sizes ranging from 59.34 to 81.09 nm. These findings confirm successful synthesis of nanoparticles within the nanoscale range.

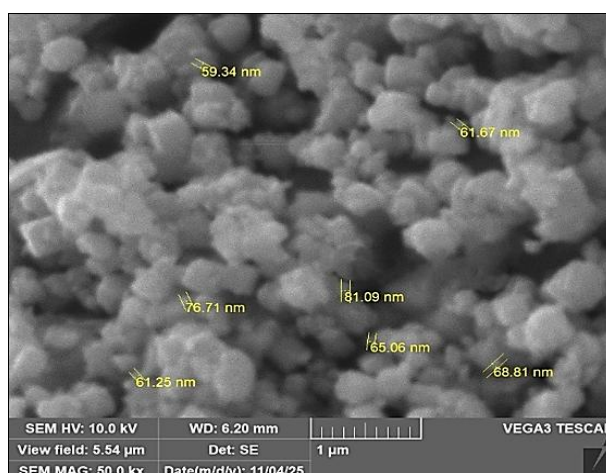


Fig 5: SEM image of chitosan coated Fe_3O_4 nanoparticles

Nanoparticles with sizes below 100 nm are reported to exhibit reduced recognition and phagocytosis by macrophages, thereby enhancing their suitability for biological applications (Stolnik *et al.*, 1995) ^[13]. Additionally, smaller particle sizes are associated with improved cellular adhesion, as attachment efficiency decreases with increasing particle size (Patil *et al.*, 2001) ^[11]. The rough and non-uniform surface morphology observed in the present study may be attributed to the chitosan coating, which is known to influence surface characteristics and nanoparticle-cell interactions. SEM analysis, which enables direct visualisation of nanoparticle morphology through detection of secondary electrons, confirmed the

formation of chitosan-coated IONPs with favourable size and surface features (Jores *et al.*, 2004) ^[4].

Chemical structure analysis

FTIR spectroscopy was employed to confirm the chemical composition and surface functionalisation of the nanoparticles at different stages of synthesis. In the present study, the FTIR spectrum of PNA-conjugated chitosan-coated IONPs exhibited characteristic absorption bands in the regions of 3200-3500 cm^{-1} , 2920-2850 cm^{-1} , 1650 cm^{-1} , 1540 cm^{-1} , 1020-1100 cm^{-1} , and 560-580 cm^{-1} .

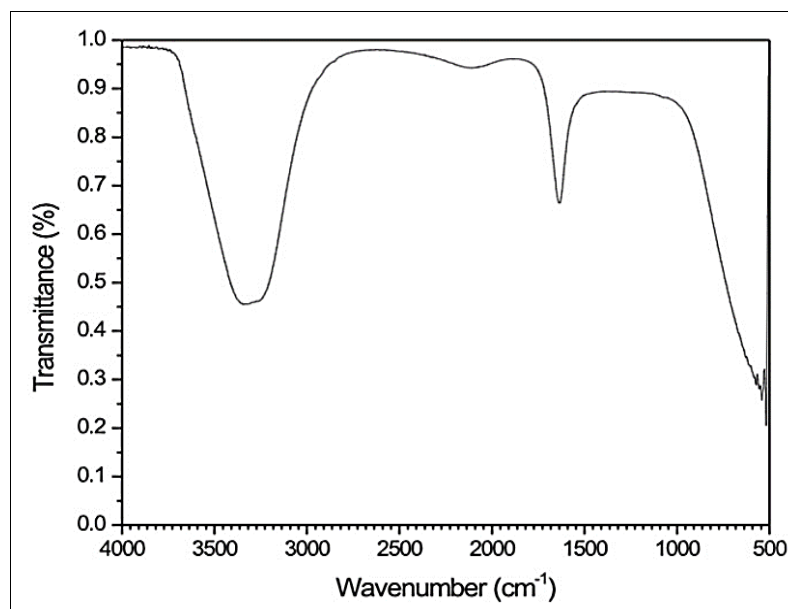


Fig 6: FTIR spectrum of PNA-conjugated chitosan-coated iron oxide nanoparticles

The absorption band observed around 560-580 cm^{-1} corresponds to Fe-O-Fe stretching vibrations, confirming the presence of IONPs, in agreement with earlier reports on magnetite nanoparticles (Yousef *et al.*, 2020) ^[14]. Following chitosan coating, the appearance of absorption bands near 1028 cm^{-1} , attributed to C-O stretching vibrations, indicated successful surface modification. After lectin immobilisation, increased absorption intensity in the amide I and amide II regions was observed, corresponding to C=O stretching and C-N stretching coupled with N-H bending vibrations, respectively. These spectral features collectively confirmed effective coating and subsequent biomolecule conjugation, consistent with previous findings (Yousef *et al.*, 2020) ^[14].

Surface charge analysis

Zeta potential is a key indicator of surface charge characteristics and colloidal stability of nanoparticle systems. In the present study, PNA-conjugated chitosan-coated IONPs exhibited a monomodal zeta potential distribution with a mean value of approximately -26 mV, indicating a predominantly negatively charged and relatively homogeneous nanoparticle surface.

Surface charge influences electrostatic interactions between

nanoparticles and the surrounding biological environment, thereby affecting dispersion stability and biological performance (Pangi *et al.*, 2003) ^[9]. Zeta potential reflects the magnitude of electrostatic repulsion between particles, with lower absolute values associated with an increased tendency for aggregation (Kumar and Dixit, 2017) ^[5]. The moderately negative zeta potential observed may be attributed to the presence of negatively charged functional groups or adsorption of hydroxyl ions following PNA conjugation, as reported in similar nanoparticle systems (Zhang *et al.*, 2016) ^[15].

Nanoparticles with zeta potential values exceeding ± 30 mV are generally considered highly stable, whereas values around ± 25 mV indicate incipient stability (Pangi *et al.*, 2003; Kumar and Dixit, 2017; Yousef *et al.*, 2020) ^[9, 5, 14]. Accordingly, the observed zeta potential of -26 mV suggests moderate electrostatic stability with potential aggregation over time. Nevertheless, the monomodal distribution and consistent surface charge confirm successful surface functionalisation and acceptable colloidal stability, supporting the suitability of these nanoparticles for further biological applications.

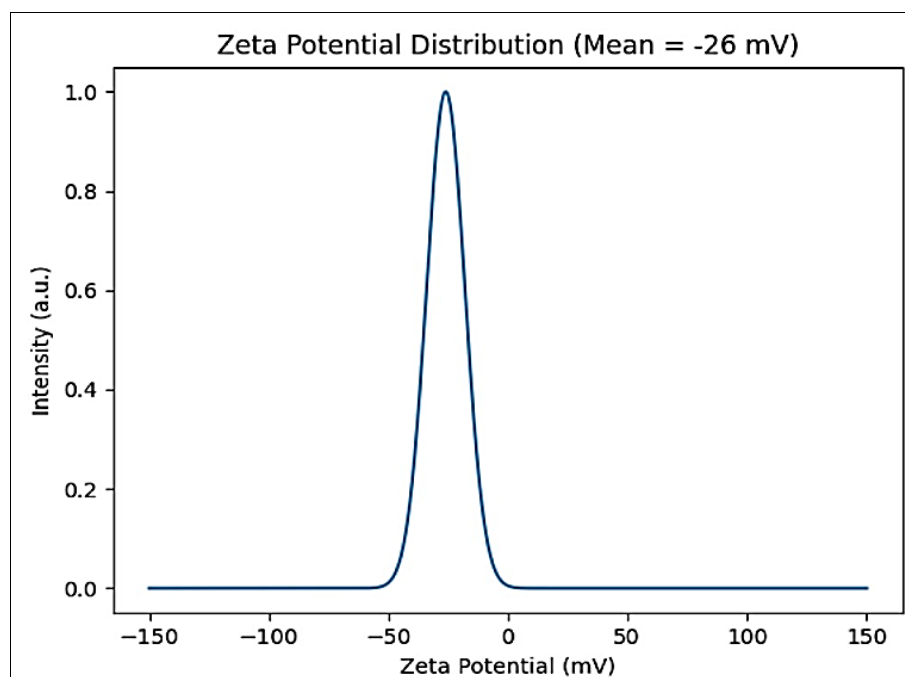


Fig 6: Zeta potential of PNA-conjugated chitosan-coated iron oxide nanoparticle nanoparticles

Conclusion

The present study successfully demonstrated the synthesis, surface modification, and physicochemical characterisation of PNA-conjugated chitosan-coated IONPs using complementary analytical techniques. UV-visible spectroscopy confirmed the formation and optical stability of IONPs and the preservation of the iron oxide core following chitosan coating and biomolecule conjugation. SEM analysis revealed nanoparticles predominantly within the nanoscale range with favourable morphology and surface features relevant for biological applications. FTIR spectroscopy validated effective chitosan coating and subsequent PNA conjugation through the presence of characteristic functional group vibrations. Zeta potential analysis indicated a moderately negative surface charge with monomodal distribution, reflecting acceptable colloidal stability and successful surface functionalisation. Collectively, these findings confirm the successful development of a structurally stable and functionally modified nanoparticle system, supporting its suitability for further biological and biomedical applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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