1. Introduction

Over recent decades, nanomaterials have emerged as promising tools for use in disease diagnosis [1]. In particular, gold nanoparticles (AuNPs)[1] hold great potential in biosensing applications because of their ease of functionalisation, high surface-area-to-volume ratio, unique bio-catalytic abilities, and high stability. AuNPs are employed in a wide range of biomedical applications including drug-delivery, bio imaging and as optical indicators in lateral flow assays, whilst their intrinsic peroxidase-like activity has facilitated their use in enzyme-like mediated biocatalysis of common ELISA substrates including 3,3',5',5-tetramethylbenzidine (TMB) [2]. Similarly, magnetic nanoparticles (MNPs) which can be manipulated and controlled by magnetic fields have been used as catalysts in bioimaging and biomedicine [3]. MNPs can have dual functionality: their magnetic properties facilitate bio-separation of target antigens; and they can biocatalyse due to the surface charge associated catalytic activity conferred by their external surface shell [4]. Combining two different nanomaterials in a composite NP structure has led to the development of bi-functional NPs including metallic magnetic iron oxide core – gold shell NPs which are capable of magnetic separation and are biocompatible [5-7].

Tuberculosis (TB) is a disease caused by a group of organisms collectively called the *Mycobacterium tuberculosis* complex (MTBC). Over one-quarter of the world's population is infected with MTBC organisms and TB is the leading cause of death worldwide from a single infectious agent [8]. *Mycobacterium tuberculosis* (MTB) is the primary cause of human tuberculosis (HTB), however, *Mycobacterium bovis* can also cause TB in humans, referred to as zoonotic TB (ZTB) [9]. TB disease caused by *M. bovis* is clinically and pathologically indistinguishable from TB caused by MTB. In addition, both mycobacteria stain as acid fast

[1] *Abbreviations:* MTBC, *Mycobacterium tuberculosis* complex; Au-Fe_3O_4 NPs, Ferromagnetic gold nanoparticles; Fe_3O_4 NPs, iron oxide nanoparticles; TMB, 3,3',5',5-tetramethylbenzidine; oxTMB, 3,3',5,5'-tetramethylbenzidine diamine; PBS, Phosphate-buffered saline; NPIDA, nanoparticle based immune detection assay; MTB, *Mycobacterium tuberculosis*; mAb, monoclonal antibody; AuNPs, gold nanoparticles; MNPs, magnetic nanoparticles; ZTB, zoonotic TB; HTB, human tuberculosis; CDC, Centers for Disease Control and Prevention; WHO, World Health Organisation; NAATs, nucleic acid amplification tests; HRP, Horseradish peroxidase; QUBPA-1, MTBC selective polyclonal antibody; QUBMA-1, *M. bovis* selective monoclonal antibody

bacilli, are 99.95% similar at the genome level, and have identical 16S rRNA sequences [10]. Differentiation between them is crucial as *M. bovis* is intrinsically resistant to pyrazinamide, one of the routinely used front-line anti-TB drugs [11]. In high TB burden, low-income countries, speciation of the causative organisms is rarely undertaken, with the result that when TB is diagnosed it is assumed to be caused by MTB [12]. Inaccurate diagnosis of ZTB compromises the efficacy of the treatment regime. This increases the risk of disease transmission and potentially contributes to the development of antibiotic-resistant TB, resulting in increased patient morbidities and mortalities [13]. Differential diagnosis of the causative agent of TB infection is therefore a crucial part of effective disease control plans to: (i) effectively direct chemotherapy; (ii) facilitate the study of the transmission of mycobacteria between humans and animals; and (iii) provide more accurate estimations of the scale of ZTB. The “WHO End TB Strategy” details targets, including an 80% reduction in TB incidence and 95% decrease in TB mortality by 2030, by prioritising patient-centred care strategies, expanding access to TB treatments for all patients, and calling for diagnosis and treatment of every person with TB, including ZTB [14]. Therefore, the development of more rapid and easy-to-use testing approaches for TB detection and speciation are necessary.

The gold standard for TB diagnosis is the isolation of MTB by culture, but this is expensive and time-consuming because of the slow-growing nature of MTBC organisms, which can take weeks for colonies to grow [15]. In low-income countries, TB diagnosis relies mainly on the detection of acid-fast bacilli using sputum smear microscopy, but this method is non-specific and has low sensitivity [16]. Molecular diagnostic assays including Nucleic Acid Amplification Tests (NAATs) [17], and the WHO endorsed GeneXpert MTB/RIF [18], ELISA [19] and other immunological tests such as the T-SPOT and QuantiFERON TB IFN-γ release assays [20] offer more sensitive diagnosis than conventional sputum smear microscopy, but suffer from limitations including requirements for highly skilled personnel, expensive lab equipment and complex experimental procedures. In addition, none of these tests can differentiate between MTB and *M. bovis*.

The slow-growing pathogenic mycobacteria use a variety of strategies thought to influence immunopathogenicity. The most important of these tools are secreted proteins, such as the 6-kDa early secreted antigenic target (ESAT6) [21], the 10-kDa culture filtrate protein (CFP10) [22], the antigen 85 (Ag85) complex which has a significant role in the virulence of MTB [23], and MPT64, which is a secreted protein involved in inhibition of macrophage apoptosis [24]. Detection of these proteins can be used to differentiate MTBC from the non-TB producing mycobacteria, for example, the detection of the MPT64 antigen has been used in various assays such as the SD Bioline [25], Becton Dickinson MGIT TBc ID test [26] and Tauns Capilia TB-Neo [27]. These diagnostic tests will identify MTBC, but will not speciate *M. bovis*. Differentiation of MTB and *M. bovis* has been demonstrated using molecular based methods which target the *gyrB* (Rv0005) [28] and *hupB* (Rv2986c) [29] gene loci. The former is a two-step approach using an AuNP-based colorimetric nanoprobe assay for the rapid detection of MTB complex, and differentiation of *M. bovis* and MTB using *gyrB* (Rv0005) target [28]. On the other hand, Prabhakar *et al* reported a PCR based assay for distinguishing MTB and *M. bovis* by targeting the *hupB* gene in a single reaction [29]. Three primers—N, M, and S—were designed and the primer pair N-S amplified the entire *hupB* gene. The C-terminal part of the gene was selectively amplified by using primers M and S and the size differences in PCR products were observed to be reliable for distinguishing MTB and *M. bovis* from other MTBC members. In a previous study, focused in the veterinary context where *M. bovis* predominates, an *M. bovis* cell wall-associated protein was used to develop a lateral flow device capable of detecting *M. bovis* [30, 31]. For translation into the human diagnostic context, where both MTB and *M. bovis* are encountered, a dual test is required that can both identify MTBC and differentiate *M. bovis*. In this study we produced a novel recombinant antibody to MPT64 and combined this with previously produced antibodies to develop a low-cost, portable and highly sensitive NP based assay. The combination of antibodies used resulted in an immunoassay which could both detect MTBC and differentiate *M. bovis*. This novel assay, when compared with conventional ELISA using the same antibodies, was found to achieve greater physiochemical stability, catalytic efficiency, and a shorter time to detection of MTBC

organisms. The magnetic capabilities of the functionalised Au-Fe_3O_4 NP bioconjugates were exploited during sample preparation, and their intrinsic peroxidase-like catalytic activity resulted in a semi-quantitative colorimetric detection methodology which directly confirms presence/absence of MTBC organisms. The developed assay enabled simultaneous immunological sensing and differentiation of *M. bovis* and MTB. No cross-reactivity was detected with other non-tuberculosis *Mycobacterium spp.* tested. This novel immunoassay combined sample preparation and target detection, reduced the number of steps, and therefore the time required to detection, when compared to traditional ELISA technique.

2. Methods

2.1 *Materials*

TMB was purchased from Merck-Millipore, hydrogen peroxide (H_2O_2), sodium acetate (Na Ac), sodium bicarbonate, Tween 20, horseradish Peroxidase (HRP), phosphate-buffered saline (PBS), sulphuric acid (H_2SO_4), dithiothreitol (DTT), Fe (II) and Fe (III) chloride, tetramethylammonium hydroxide (TMOH), sodium citrate ($Na_3C_6H_5O_7$) and tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich. MPT64 antigen was purchased from 2BScientific Ltd (Heyford, Oxfordshire, UK). Costar high-binding 96-well plates were purchased from Corning (New York, USA), 96-well polypropylene microtiter plates were purchased from Greiner Bio-One (Kremsmünster, Austria), DAKO Goat anti-rabbit and anti-mouse HRP-conjugated antibodies were purchased from Agilent (CA, USA). Middlebrook 7H9 broth was purchased from Becton Dickinson DIFCO (USA). PD-10 columns were purchased from GE-Healthcare. MTB H37Rv, *M. bovis* AF2122/97, *M. kansasii* NCTC10268, *M. avium* subsp. *paratuberculosis* NCTC8578 and *M. bovis* (BCG-strain) NCTC5692 cell suspensions were produced in-house as detailed previously [30]. Briefly, enumerated bacterial cultures were centrifuged and washed three times in sterile PBS (0.1M, pH 7.4) before resuspension in PBS. All mycobacterial cell suspensions were subjected to a 10 kGy dose of gamma radiation (Gammabeam 650 cobalt irradiator, AFBI, Belfast). After radiation treatment, samples of each suspension were cultured on Middlebrook 7H10 agar and plates read after

56 days, to ensure complete inactivation of all pathogenic mycobacteria before use. Irradiated bacterial stock cultures diluted in PBS (10^6 CFU mL^{-1}) were stored at -80 ^oC until required. *Escherichia coli* TG1 DSM6056, and XL1-Blue MRF' strains were cultivated in house.

2.2 *Antibody production*

An *M. bovis* mAb and MTBC polyclonal antibodies, labelled QUBMA-1 and QUBPA-1 respectively were sourced from a previous study [32].

2.2.1 *Recombinant antibody selection via phage display*

Recombinant antibodies against MPT64 were generated by antibody phage display. The antibody selection was performed as described previously with modifications and the resultant monoclonal binders were sequenced and analysed using VBASE2 (www.vbase2.org) [33-35]. (See S1)

2.2.2 *Initial characterisation*

Unique single-chain variable fragment (scFv) sequences isolated by antibody-phage display in Costar high-binding 96-well plates were subcloned into pCSE2.6-mIgG2a-Fc-XP [36] using *Nco*I/*Not*I (New England Biolabs, Frankfurt, Germany). For production, the transfected EXPI293F cells were cultured in chemically defined medium F17 (Thermo Fisher Scientific) supplemented with 0.1% pluronic F68 (PAN-Biotech, Aidenbach, Germany) and 7.5 mM L-glutamine (Merck). A subsequent protein A purification was performed as described previously [37].

The purified scFv-Fc antibodies were initially characterised by indirect ELISA, performed by coating 50 ng per well of MPT64 diluted in PBS. The recombinant antibodies were 10-fold serially diluted in 2% Modified PBS-T (2% (w/v) milk powder in PBS; 0.05% Tween20) (MPBS-T) from 10 µg mL^{-1} to 3.16 ng mL^{-1}, and 100 µL per well of each dilution added. The secondary antibody, goat anti-mouse HRP, was added and the reaction developed with TMB solution and read at 450 nm with a 620 nm reference. As a negative control, a non-related His-tagged

recombinant protein produced in *E. coli* was coated on a plate and tested in the same way. Absorbance values were used to calculate EC_{50} value using GraphPad software (Prism, v 5.01).

2.3 *Synthesis of Fe_3O_4 and Au-Fe_3O_4 composite NPs*

The method of Pham *et al* was used to synthesise Fe_3O_4 NPs [38]. Briefly, ddH_2O (20 mL) was degassed by sonicating for 30 min in a triple neck flask before purging with N_2 gas for 20 min. Fe (III) chloride (4.6g) and Fe (II) chloride (1.7g) were added to HCl (100 mM) and stirred vigorously until fully dissolved before adding NaOH (2 M, 20 mL) dropwise yielding a dark black magnetic precipitate. The precipitate was collected using a 3,000 G magnet and washed in ddH_2O before resuspension in tetramethylammonium hydroxide (TMOH) (0.1 M, 10 mL). Iron oxide NPs were obtained by further washing and dissolving in nitric acid (HNO_3) (0.01 M, 10 mL) with stirring until the solution produced a brown colour. The synthesised iron oxide NPs were washed in ddH_2O before resuspending in TMOH (0.1 M, 10 mL).

A modified version of the Turkevich method for AuNP production in trisodium citrate [39] was used to synthesise gold-iron oxide nanocomposites. Sodium citrate (0.1 M, 10 mL) and synthesised iron oxide NPs (1 mL) were boiled with stirring for 10 min, before addition of $HAuCl_4 \cdot 4H_2O$ (1 mM, 1 mL) to produce iron oxide-gold composite NPs. A red-wine colour developed within 10 min and the resultant composite NPs were magnetically collected and washed three times in ddH_2O (10 mL), and once in sodium citrate (0.1 M, 10 mL), before storing at 4 °C until use. The resultant composite NPs were standardised to OD_{550} = 1.0 before use.

2.4 *Characterisation of Au-Fe_3O_4 NPs*

The peroxidase activity of the Au-Fe_3O_4 NPs was assessed at a range of H_2O_2 concentrations, pH and temperatures. Au-Fe_3O_4 NPs (0.1 nM, OD_{550} = 1) and HRP (3 nM) solutions were prepared in sodium acetate buffer (2 mM, pH 5, 1 mL). The peroxidase activity of Au-Fe_3O_4 NPs and HRP was compared by incubating 1 mL of each Au-Fe_3O_4 NP solution and HRP in

sodium acetate buffer with TMB (0.65 mM, 100 µL) for 2 hrs at 37 ^{o}C. The peroxidase activity was measured at a range of: (i) H_2O_2 concentrations, by preparing solutions of Au-Fe_3O_4 NP (1 mL) at final H_2O_2 concentrations of 0, 1, 2, 3, 4, 6, 8 and 10% (v/v), (ii) pH, by preparation of Au-Fe_3O_4 NP solutions (1 mL) with pH adjusted to 2, 3, 4, 5, 6, 7, 8 and 9 and, (iii) temperatures, by varying the incubation temperature of five Au-Fe_3O_4 NP solutions (1 mL) to 20, 30, 40, 55 and 65 ^{o}C. TMB substrate oxidation absorbance readings were measured using a Safire 96-well ELISA plate reader (Tecan, Switzerland) at 370 nm immediately after incubation.

TMB oxidation was demonstrated by preparing two solutions of 0.65 mM TMB / 8% (v/v) H_2O_2 in sodium acetate buffer (2 mM, pH 5, 1 mL) in semi-micro cuvettes. Au-Fe_3O_4 NPs (0.1 nM, 10 µL) were added to one cuvette and the TMB substrate oxidation profile recorded in both cuvettes using a spectrophotometer.

To determine if the relative peroxidase activity being measured was due to intact Au-Fe_3O_4 NPs, two solutions of Au-Fe_3O_4 NPs (0.1 nM) in sodium acetate buffer (2 mM, pH 5, 1 mL) were prepared. A 3000 G permanent magnet was used to remove the Au-Fe_3O_4 NPs from one solution. One hundred µL of 0.65 mM TMB / 8% (v/v) H_2O_2 was added to both and the relative peroxidase activity of each solution was measured continuously for 10 min at 370 nm using a spectrophotometer.

2.5 *Characterisation of NP structure*

Transmission electron microscopy images of Au-Fe_3O_4, Au and Fe_3O_4 NPs were obtained using a Joel JEM-1400 Plus Transmission Electron Microscope 120 kV. Samples were prepared on carbon-nickel grids in 50% (v/v) ethanol. UV-Vis analysis of particles was achieved using a Cary 60 spectrophotometer (Agilent Technologies, USA). Dynamic Light Scattering (DLS) particle size and zeta potential measurements were obtained using a Malvern Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK)

2.6 *Au-Fe_3O_4 NP functionalisation and characterisation*

QUBPA-1 was used as the capture antibody in the assay. Au-Fe_3O_4 NPs were bio-functionalised by direct coupling of QUBPA-1 using DTT [40-42]. Briefly, DTT (0.35 mg) was added per 1 mL of antibody (0.2 mg mL^{-1}) diluted in PBS EDTA (2 mM, pH 7.4, 1 mL) buffer solution, and incubated on a rotator for 30 min at room temperature. Excess reactants were removed by separation in PD-10 columns using PBS EDTA washing buffer before collecting eight successive eluted antibody fractions (0.5 mL). Antibody fraction concentrations were measured at 280 nm on a NanoDrop 8000 (Thermoscientific, UK) and the peak antibody fraction (0.1 mg mL^{-1}) was added to 1 mL of Au-Fe_3O_4 NPs (OD_{550} = 1) and left for 16 h at room temperature to enable conjugation. Resultant antibody Au-Fe_3O_4 NP bioconjugates were washed three times in PBS-0.05% Tween-20 buffer (pH 7.4) (PBS-T) and stored at 2 – 8 ^oC. Relative peroxidase activity of uncoated and antibody functionalised Au-Fe_3O_4 NPs was determined by preparing solutions of uncoated Au-Fe_3O_4 NPs and antibody functionalised Au-Fe_3O_4 NPs (0.1 nM) in sodium acetate buffer (2 mM, pH 5, 1 mL). TMB (0.65 mM) / 8% (v/v) H_2O_2 (100 µL) was added to each sample and the relative peroxidase activity of each solution measured continuously for 10 min at 370 nm using a spectrophotometer.

2.7 *Immunoassays*

2.7.1 NP-based Immune Detection Assay (*NPIDA)*

Nunc Maxisorp™ plates were coated with detector antibodies, either recombinant antibody or QUBMA-1 (10 µg mL^{-1}, 100 µL), diluted in bicarbonate buffer (0.1 M, pH 9.4), and incubated for 16 h at 4 °C. After incubation, they were washed five times with PBS-T wash buffer before blocking with assay buffer (PBS-T/BSA (1% w/v)), (200 µL per well) for 2 h at 37 °C with shaking. The solution was then discarded.

Concurrently, Au-Fe_3O_4 NP-antibody bioconjugates (100 µL), coated with the capture antibody, were incubated with whole cells of either MTB or *M. bovis* (1 x 10^5 CFU mL^{-1}) diluted in assay buffer (1 mL) for 16 h at 37 °C in 1.5 mL tubes. Negative control samples were prepared by incubating Au-Fe_3O_4 NP-antibody bioconjugates (100 µL) with assay buffer only. After incubation with target cells, Au-Fe_3O_4 NP-antibody bioconjugates were washed three

times in PBS-T/SDS (0.001 % w/v), collected using a magnetic stand between each wash. The washed bioconjugate was then added to the antibody coated, blocked plate, and incubated for 2 h at 37 °C with shaking. The plates were washed five times using PBS-T before adding TMB substrate (100 µL per well). The colour was left to develop for 30 min, stop solution (2.5 M H_2SO_4, 25 µL per well) added, and the plate read at 450 nm using a microplate reader.

2.7.2 *Conventional sandwich ELISA*

For comparison with conventional sandwich ELISA the same method was followed as detailed previously for the NPIDA with minor changes. Nunc Maxisorp™ plates were coated as before. After removing blocking buffer and washing plates five times with PBS-T, each target bacterial culture (1 x 10^5 CFU mL^{-1}, 100 µL per well) diluted in assay buffer, was added directly to the wells and incubated for 2 h at 37 °C with shaking. Plates were washed five times with PBS-T, the detector antibody (either QUBPA-1, GSM237-G8 or QUBMA-1) (10 µg mL^{-1}, 100 µL per well) added, and the plates incubated for 1.5 h at 37 °C. Plates were washed five times with PBS-T and the appropriate secondary antibody, (DAKO anti-mouse/ rabbit HRP conjugate antibody) (0.25 µg mL^{-1}, 100 µL per well), diluted in assay buffer added, and incubated for 1 h at 37 °C with shaking. Plates were then washed 5 times in PBS-T before adding TMB substrate (100 µL per well). The plate was left for 30 mins before adding stop solution, (2.5 M H_2SO_4, 25 µL per well) and the absorbance read at 450 nm using a microplate reader.

2.7.3 *Limits of Detection of developed assays*

The 50% limit of detection ($LOD_{50\%}$) for both the NPIDA and the conventional sandwich ELISA were determined both in assay buffer and in BACTEC™ MGIT™ TB media (7H9 Middlebrook broth/10 % OADC) using the assay procedures detailed above. A tenfold dilution series containing 10^5, 10^4, 10^3, 10^2, 10^1 and 0 CFU mL^{-1} of both MTB and *M. bovis* whole cells was prepared in assay buffer and in BACTEC™ MGIT™ TB media. In the NPIDA, each cell dilution (1 mL) was incubated with Au-Fe_3O_4 NP-antibody bioconjugates, whereas in the conventional

sandwich ELISA each cell dilution was added to a previously coated 96-well plate (recombinant antibody or QUBMA-1) after the blocking step. All samples were prepared in triplicate, all experiments were repeated three times resulting in a total of 9 replicate values at 6 dilutions. The $LOD_{50\%}$ was determined using the generalized Spearman-Kärber $LOD_{50\%}$ calculation for 6-level spiking protocols [43].

3. Results and Discussion

In this study, a novel ferromagnetic gold NP immunoassay was developed and evaluated for the detection and differentiation of MTB and *M. bovis*, the main causative agents of TB in humans. A unique recombinant monoclonal antibody to a key MTBC protein was generated and used in conjunction with a panel of pre-existing antibodies to cell surface and secreted antigens of MTB and *M. bovis,* to develop the assay. Au-Fe_3O_4 NP bioconjugates were prepared by direct coupling of antibodies to Au-Fe_3O_4 NPs which were then used to develop the NPIDA. The NIPIDA was capable of sensitive detection and differentiation of MTB and *M. bovis* cells.

3.1 *Au-Fe_3O_4 NP composition and peroxidase-like activity*

Fe_3O_4 NPs were synthesised first, and subsequently alloyed with Au to form Au-Fe_3O_4 NPs following a modified version of the established sodium citrate seeding method by Brown *et al* [44]. As seen in **Figure 1a**, Fe_3O_4 NPs do not exhibit any peaks from 400-800 nm (green line), and AuNPs possess a characteristic plasmonic peak at 517 nm (solid line). The formation of Au-Fe_3O_4 NPs was confirmed by the UV-Vis spectroscopy with the presence of a distinct peak at 550 nm corresponding to the surface plasmonic peak of the Au nanomaterial. The shift in peak wavelength (from 517 to 550 nm) corresponded to an increase in NP diameter. This has previously been observed during Au-Fe_3O_4 NP synthesis, wherein successful assembly of Au with the Fe_3O_4 NPs led to a similar shift in peak wavelength [45]. The magnetic capabilities of Fe_3O_4 NPs and Au-Fe_3O_4 NPs were demonstrated by collection using a 3000 G permanent magnet, resulting in a phase separation in both the Fe_3O_4 NP and Au-Fe_3O_4 NP solutions after

the ferromagnetic NPs had migrated towards the permanent magnet (**Figure 1b**). The demonstration of both magnetism and plasmonic properties unambiguously confirms the successful production of Au-Fe_3O_4 NPs. TEM images further revealed that the multiple-faced Fe_3O_4 NPs and the Au-Fe_3O_4 NPs composites have estimated diameters ranging from 15 – 50 nm (**Figure 1c**). Pham *et al* also reported similar observations during Au-Fe_3O_4 NP synthesis, noting the formation of spherical-like, multiple-faced Au-Fe oxide NPs following the reduction of Au^{3+} ions onto 'jagged' Fe oxide NPs [38]. DLS measurements were taken for Fe_3O_4 NPs and Au-Fe_3O_4 NPs in liquid phase and revealed larger nanoparticle hydrodynamic diameters, i.e. 153.7 and 300.6 nm, respectively **(Figure S1a)**. The discrepancy between the estimated diameters obtained by TEM and by DLS is thought to be as a result of the magnetic nature of the iron oxide component of the nanomaterials which causes them to magnetise and agglomerate into larger particle clusters, an effect which has also been reported by other groups during analysis of Au-Fe_3O_4 NPs [46, 47]. In fact, the hydrodynamic diameter of the same Au-Fe_3O_4 NPs increased from ca. 300 nm to 800 nm as a function of time **(Fig S1b)** due to the magnetised susceptibility of the Au-Fe_3O_4 NPs. The agglomeration causing apparent increased particle size **(Fig S1c)** can be reversed by applying a prompt sonication (data not shown).

3.2 *Functionalisation of the Au-Fe_3O_4 NPs forms the basis of a novel NP-based immunoassay*

The intrinsic peroxidase-mimicking ability of Au-Fe_3O_4 NPs enables them to perform as an artificial-HRP nanozyme capable of oxidising TMB in the presence of H_2O_2. The catalytic efficiency of enzyme catalysts such as HRP and inorganic nanozymes, like Au-Fe_3O_4 NPs, is dependent on environmental conditions including temperature, pH and H_2O_2 concentration. Inorganic nanomaterials, such as Au-Fe_3O_4 NPs, are predicted to have greater thermal, pH and chemical tolerances than organic enzymes like HRP [48]. To assess this hypothesis, consecutive experiments were set up, measuring TMB oxidation at fixed concentrations of 3 nM HRP and 0.1 nM Au-Fe_3O_4 NP respectively, under varying environmental conditions

including H_2O_2 concentration, temperature and pH. Au-Fe_3O_4 NPs were found to require H_2O_2 concentrations of 8 % (v/v) to achieve maximal activity and were inhibited at lower/ higher concentrations, whereas, for HRP the optimal H_2O_2 concentration was determined to be 0.5 % (v/v) which decreased rapidly at increasing concentrations (**Figure 2a**). Both Au-Fe_3O_4 NP and HRP exhibited optimal catalytic activity around pH 5, however the Au-Fe_3O_4 NPs demonstrated greater peroxidase activity over a broader range of pH values, pH 4 to pH 7, compared to HRP (**Figure 2b**). At temperatures over 37 ^oC, HRP catalytic activity was 25% lower than Au-Fe_3O_4 NPs (**Figure 2c**). The maximum point of each curve was set as 100% relative activity and optimal conditions for TMB oxidation were determined to be 8% H_2O_2, 37 ^oC at pH 5. The oxidation of TMB (0.65 mM) by Au-Fe_3O_4 NPs in the presence of 8% H_2O_2 produces a blue oxidation product, whilst in the absence of Au-Fe_3O_4 NPs, TMB is unoxidised and the solution remains colourless (**Figure 2d**). TMB oxidation produces two major peaks observed at 370 nm and 652 nm by Au-Fe_3O_4 NPs corresponding to the formation of blue, oxidised TMB product (**Figure 2e**) [49]. Previous studies have highlighted the peroxidase-like activity of leached Fe (II) and Fe (III) ions in acidic solution [7, 48]. Therefore, to confirm that the observed peroxidase-like activity is due to intact Au-Fe_3O_4 NPs, and not leached iron ions, a 3000G magnet was used to remove Au-Fe_3O_4 NPs and the remaining solution analysed along with the original preparation of Au-Fe_3O_4 NPs. TMB was added to each sample and relative peroxidase activity of both solutions measured at 370 nm for 10 min. In the presence of Au-Fe_3O_4 NPs, TMB oxidation increased steadily over 10 min, producing the blue coloured oxidised TMB product, whereas in samples with Au-Fe_3O_4 NPs removed (No-NP), TMB remained unoxidised and the sample remained colourless (**Figure 2f**). The peroxidase-like activity was therefore due to intact Au-Fe_3O_4 NPs which represent potential candidates for oxidation of TMB in environmental conditions unsuitable for conventional organic enzymes like HRP.

Modification of NPs with small ligands and metal ions has often been reported to result in a decrease of catalytic activity. For example, Tao *et al* [50], reported a decrease in peroxidase-like activity of AuNPs as a result of hindrance by the small ligand biomolecule dopamine.

Likewise, antibodies may cause a decrease in peroxidase activity by surface hindrance. In this study, we compared the relative peroxidase activity of uncoated and antibody-functionalised Au-Fe_3O_4 NPs (OD_{550} = 1) (**Figure 3**). After 10 mins the rate of formation of blue oxidised TMB product was reduced by approximately 25 % following surface attachment of the antibody to the Au-Fe_3O_4NPs (uncoated Au-Fe_3O_4 NPs Abs = 1.04 OD_{370}, antibody-functionalised Au-Fe_3O_4 NP Abs = 0.72 OD_{370}) (**Figure 3a**). However, despite this suppression of catalytic activity the resultant bioconjugate could still be used for direct sample sensing. The UV-Vis spectral analysis for TMB oxidation showed a decrease in peak absorbance at 370 nm and 652 nm, corresponding to the suppression of TMB oxidation by the antibody functionalised Au-Fe_3O_4 NPs (**Figure 3b**).

Having characterised the activity of the individual constituents they were then combined with antibodies to develop the NPIDA. In the first step the antibody-functionalised Au-Fe_3O_4 NP bioconjugate was incubated with target cells. After collecting the Au-Fe_3O_4 NP bioconjugate-cell complex on a magnet, the bioconjugate-cell complex was added to wells pre-coated with detector antibody then washed after incubation to remove unbound bioconjugate-cell complexes. TMB substrate was added to each well and the peroxidase-mimicking activity of any captured Au-Fe_3O_4 NP bioconjugate produced a blue coloured oxidation product in the presence of H_2O_2. The absorbance intensity of the oxidised TMB product was proportional to the amount of Au-Fe_3O_4 NP bioconjugate-cell complexes present after binding to the detector antibody. The more Au-Fe_3O_4 NP bioconjugate-cell complexes bound to the detector antibody, the greater the overall intensity of TMB oxidation signal produced. A schematic of the NPIDA is detailed in **Figure 4**. The NPIDA was then compared with conventional sandwich ELISA wherein plates were coated with capture antibody before adding target cells, followed by a secondary (detector) antibody, whose presence is then detected using an HRP conjugated anti-species antibody in a two-step consecutive process.

3.3 *Discrete combinations of monoclonal and polyclonal antibodies enabled speciation of MTB and M. bovis*

A total of 10 phage display derived recombinant binders were selected for investigation, produced as scFv-Fc antibodies, and initially tested by indirect ELISA. After titration using the MPT64 antigen as target and determination of cross reactivity, a single recombinant antibody (GSM237-G8) was chosen for further experiments (**Figure 5**).

Approaches which combine immunomagnetic separation with sandwich ELISA format immunoassays are more sensitive than indirect ELISA as they facilitate enrichment of groups of target cells in samples of low concentration, such as the detection and enrichment of bacterial species in wastewater samples [51]. To select the optimal combination of antibodies for detection of each organism in the NPIDA, various pairs of target-binding capture and detection antibodies were screened by conventional sandwich ELISA **(Figure S2)**. Each antibody combination was evaluated for optimal detection of MTB or *M. bovis* cells. In the conventional sandwich ELISA, the capture antibody selected for MTB was GSM237-G8, QUBMA-1 was selected for capture of *M. bovis* cells, and QUBPA-1 was selected as the detector antibody for both mycobacteria **(Figure S3)**. In the NPIDA the detector antibody selected for MTB cell detection was GSM237-G8, QUBMA-1 was selected for detection of *M. bovis* cells, and QUBPA-1 was the capture antibody selected for both mycobacteria **(Figure S4).** Au-Fe_3O_4 NPs were functionalised with QUBPA-1 by DTT reduction. This method avoids modification of the antigen binding sites by reducing IgG antibody disulphide bonds within the antibody hinge region, enabling resultant thiol groups to anchor antibodies to the gold shell of the Au-Fe_3O_4 NPs [39-44, 52]. To confirm successful immobilisation of the antibody to the functionalised Au-Fe_3O_4 NPs and assess target specificity of the novel NPIDA, a cross-reactivity analysis was carried out by NPIDA and the results compared with conventional ELISA. In the NPIDA the NP bioconjugate was incubated overnight with MTB, *M. bovis* and *M. bovis* BCG-strain (NCTC5692) and two non-tuberculosis mycobacterial species, *M. kansasii* (NCTC10268) and *M. avium* subsp. *paratuberculosis* (MAP) (NCTC8578), whilst in parallel, GSM237-G8 antibody and QUBMA-1 were coated onto separate 96-well Nunc Maxisorp™ plates, and the assay carried out as detailed previously (2.7.1). The results showed that when GSM237-G8 was used as the detector antibody the mean OD_{450} of Au-

Fe_3O_4 NP QUBPA-1 with MTB (1±0.038) was significantly higher ($P < 0.001$) than the mean value for *M. bovis* (0.33±0.014), confirming that the antibody had been successfully conjugated to the Au-Fe_3O_4 NP and the assay was more selective for MTB than *M. bovis* (**Figure 6a**).

Other research groups have reported a decrease of binding affinity resulting from conjugation of antibodies to labels such as fluorophores/ AuNPs and have indicated that such 'trade-offs' may be necessary during attachment of binders [53]. However, in the present study, target selectivity of the antibody was retained following conjugation to the Au-Fe_3O_4 NP by DTT reduction, which has also been found by Ji *et al* [41]. A comparison between conventional sandwich ELISA and Au-Fe_3O_4 NP QUBPA-1 bioconjugate with QUBMA-1 coated onto 96-well plates indicated the bioconjugate had retained its selectivity for *M. bovis* and the mean OD_{450} of Au-Fe_3O_4 NP QUBPA-1 bioconjugate with *M. bovis* (1±0.005) was found to be significantly higher ($P < 0.001$) than the mean value for MTB (0.28±0.022) (**Figure 6b**). The novel NPIDA based assays for MTB and *M. bovis* can be run simultaneously using QUBPA-1 as the capture antibody and Nunc plates which are coated on one half with GSM237-G8 to detect MTBC, and on the other half with QUBMA-1 to detect *M. bovis*. As such the one immunoassay can be used to simultaneously detect and differentiate *M. bovis* from MTB.

The BD BACTEC™ MGIT™ is a liquid media system commonly used for cultivation of mycobacteria. To determine the potential suitability of the novel NPIDA for detection of MTB and *M. bovis* in culture media, and to compare the $LOD_{50\%}$ of each pathogen in buffer and broth using both immunoassay formats, a tenfold dilution series of MTB and *M. bovis* ranging from 1 x 10^5 CFU mL^{-1} to 0 CFU mL^{-1} was prepared in assay buffer and in MGIT media. The NPIDAs and conventional ELISAs were carried out as detailed previously and the results for each assay illustrated in **Table 1**. The results confirm that both the MTB and *M. bovis* ELISAs and NPIDAs have comparable $LOD_{50\%}$ values, and therefore similar sensitivities, in both buffer and BD BACTEC™ MGIT™ and indicate minimal matrix effects when the assays were transferred from buffer into culture media. The novel NPIDA assay could be applied following

culture of samples and used to replace conventional Ziehl-Neelsen staining and subsequent molecular identification when a TB BACTEC™ MGIT™ culture indicates positive.

4. Conclusion

This study reports the synthesis of Au-Fe_3O_4 NPs by combining citrate capped AuNPs and co-precipitated Fe_3O_4 NPs. The physicochemical properties of the resultant iron oxide-gold composite NPs were characterised, and their magnetic separation capabilities and intrinsic peroxidase-like activity demonstrated. Functionalisation of the Au-Fe_3O_4 NPs with MTB and *M. bovis* selective antibodies led to the development of a NP-based immune-detection assay capable of detection and differentiating MTB and *M. bovis*. To the best of our knowledge this assay represents the first immune based diagnostic test capable of differentiating between MTB and *M. bovis*. The NPIDA demonstrated comparable levels of sensitivity and catalytic activity to conventional ELISA but enhanced pH and temperature stabilities compared with conventional HRP. The magnetic capability of the iron cores was exploited in sample preparation and the intrinsic peroxidase-mimicking ability of NPs negated the requirement for a time-consuming antibody incubation step required during conventional ELISA. The NPIDA is a low cost tool which can identify MTB, and speciate *M. bovis*, and therefore has the potential to more quickly direct patient treatment regimens thus improving the efficacy of treatments and reducing the emergence of TB drug-resistant phenotypes, particularly in high TB burden countries. The methodology described in this research should not be limited to the detection of MTBC but could be employed in other biosensing applications by combining Au-Fe_3O_4 NPs with alternative binders specific for metabolites or pathogens of biological interest. Future investigations will include extended cross reactivity studies and the application of the TB NIPDA directly with sputum samples.

Conflicts of interest

There are no conflicts to declare

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Associated Supplementary Material

Recombinant antibody selection via phage display; DLS measurement of Fe_3O_4 NPs and Au-Fe_3O_4 NPs in water; Optimisation of antibody pair combinations with QUBPA-1 detection antibody; Schematic representation of the conventional sandwich ELISA with MTB and *M. bovis*; Schematic representation of the NPIDA with MTB and *M. bovis.*

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