Lab on a Chip

Cite this: *Lab Chip*, 2012, **12**, 4802–4808

www.rsc.org/loc

PAPER

Gold on paper–paper platform for Au-nanoprobe TB detection†

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Received 29th June 2012, Accepted 29th August 2012
DOI: 10.1039/c2lc40739f

Tuberculosis (TB) remains one of the most serious infectious diseases in the world and the rate of new cases continues to increase. The development of cheap and simple methodologies capable of identifying TB causing agents belonging to the *Mycobacterium tuberculosis* Complex (MTBC), at point-of-need, in particular in resource-poor countries where the main TB epidemics are observed, is of paramount relevance for the timely and effective diagnosis and management of patients. TB molecular diagnostics, aimed at reducing the time of laboratory diagnostics from weeks to days, still require specialised technical personnel and labour intensive methods. Recent nanotechnology-based systems have been proposed to circumvent these limitations. Here, we report on a paper-based platform capable of integrating a previously developed Au-nanoprobe based MTBC detection assay—we call it "Gold on Paper". The Au-nanoprobe assay is processed and developed on a wax-printed microplate paper platform, allowing unequivocal identification of MTBC members and can be performed without specialised laboratory equipment. Upon integration of this Au-nanoprobe colorimetric assay onto the 384-microplate, differential colour scrutiny may be captured and analysed with a generic "smartphone" device. This strategy uses the mobile device to digitalise the intensity of the colour associated with each colorimetric assay, perform a Red Green Blue (RGB) analysis and transfer relevant information to an off-site lab, thus allowing for efficient diagnostics. Integration of the GPS location metadata of every test image may add a new dimension of information, allowing for real-time epidemiologic data on MTBC identification.

1 Introduction

Tuberculosis (TB) is mainly caused by *Mycobacterium tuberculosis* (*M. tuberculosis*, Mtb), a member of the *M. tuberculosis* Complex (MTBC) and, according to the World Health Organisation, it remains one of the most serious infectious diseases in the world, responsible for 1.1 million deaths and 8.8 million new cases in 2010 alone.[1] Recent advances in molecular diagnostics of TB have improved the detection capability of the pathogen but many of these methods require specialised technical personnel and expensive laboratory equipment.[2–6] Several new technologies are under development, which will enable the presumptive detection of MTBC in just one to two days. Diagnostics at point-of-need is crucial to TB control as rapid identification and pathogen characterisation may allow patients to get immediate treatment that is vital in addressing this pandemic.

Recently, there have been reports on paper-based analytical systems suitable for application in diagnostics at point-of-need.[7–10] An interesting approach combines the use of inexpensive paper-based platforms and digital image analysis to screen for relevant analytes.[11] This concept makes use of quantitative colorimetric correlations using mobile cameras to digitalise results allowing the measurement of colour intensity, *i.e.* to conduct quantitative microscale bioanalysis assays without expensive laboratory equipment. Recently, Carrilho and colleagues extended this concept *via* multizone standard 384-well paper microplates to be used as an alternative to conventional multiwell plates fabricated in moulded polymers.[12,13] Paper-based plates are functionally related to plastic well plates, and offer interesting capabilities, such as (i) multiplex assays; (ii) the ability to store, mix, and combine reagents; (iii) multiple sample assaying with a single device; and (iv) the ability to capture the result in a digital image format with a generic mobile device. The fabrication process involves two core operations: printing patterns of wax on the surface of the paper and melting the wax into the paper to form complete hydrophobic barriers. Only just have paper platforms been used for gold nanoparticle based surface enhanced Raman scattering (SERS) bio-detection assays.[14,15]

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† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c2lc40739f

Despite the wide range of nanoscale systems for biomolecular assays,[16–18] those based on nanoparticles, and gold nanoparticles (AuNPs) in particular, have been the most widely used for TB diagnostics.[19–22] These AuNPs exhibit amazing properties, such as intense colours and ease of functionalisation with relevant biomolecular moieties for bio-recognition, *e.g.* antibodies and ssDNA oligonucleotides. Solutions containing AuNPs usually exhibit an intense red colour derived from the surface plasmon resonance (SPR) band centred around 520 nm; AuNP aggregation results in a red-shift of the SPR and the solution changes colour to blue.[23] The first use of such properties for the detection of *M. tuberculosis* was introduced by our group in 2006 in an assay that relied on the colorimetric changes of a solution containing AuNPs functionalised with thiol-modified ssDNA oligonucleotides (Au-nanoprobe) complementary to a region of the RNA polymerase beta subunit *locus* capable of identifying MTBC members.[20] The method relies on the hybridisation between the Au-nanoprobe and the target sequence from the pathogen. In this non-cross-linking assay, the aggregation of the AuNPs is induced by salt addition and the presence of a complementary target prevents Au-nanoprobe aggregation and the solution remains red; the absence of a complementary target does not prevent Au-nanoprobe aggregation, which results in a visible colour change from red to blue. This low-complexity assay was subsequently used for the detection of MTBC specimens and mutations associated with antibiotic resistance in clinical specimens with remarkable sensitivity in just a few hours.[20,22,24]

Here, we report on the integration of this colorimetric Au-nanoprobe assay with a paper-platform that allows colour development and a simple data analysis tool capable of the specific detection of MTBC members. The Au-nanoprobe hybridisation to the specific DNA target sequence yields an intensive red-colour that can be easily differentiated from a non-complementary sequence, which yields a blue coloured solution. The wax printed 384 well plate is impregnated with a pre-determined concentration of salt ($MgCl_2$) capable of inducing Au-nanoprobe aggregation, thus yielding a colorimetric discrimination indicating the presence of the specific DNA target. Because of the white background of the paper, the colour contrast is greatly improved without the need for expensive constituents. We further demonstrate the integration of this paper-platform with a smartphone and a simple data analysis tool that together are capable of quantifying the colorimetric changes on the paper plate system and transmitting the metadata to a centralised off-site laboratory (Fig. 1). This principle can be extended to any other standard mobile imaging device with GPS capability. This integrated *Gold on Paper* platform was then used

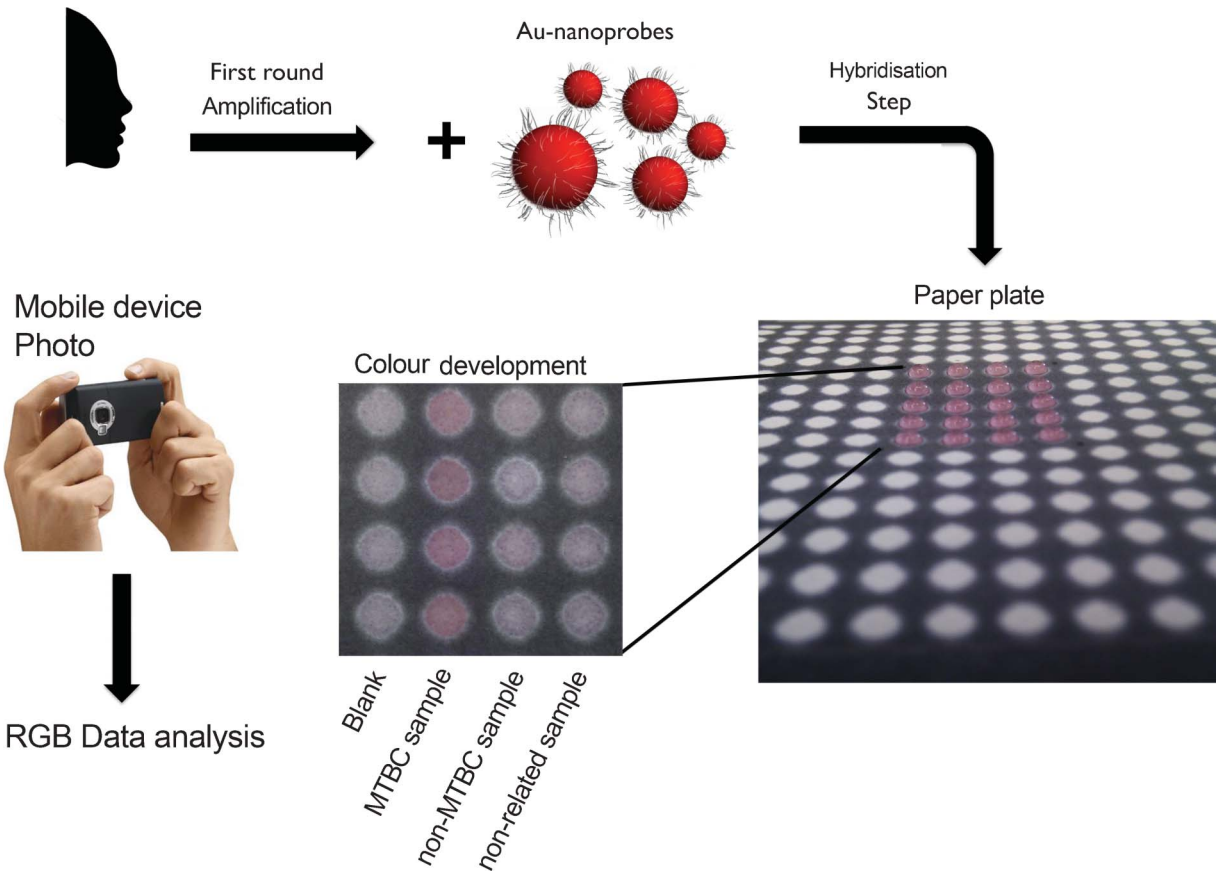

Fig. 1 Au-Nanoprobe strategy for the detection of MTBC members. Schematic representation of the detection with gold nanoprobes. The colorimetric assay consists of visual comparisons of test solutions after salt induced Au-nanoprobe aggregation on a [$MgCl_2$] impregnated paper plate: MTBC Au-nanoprobe alone—Blank; MTBC Au-nanoprobe in the presence of MTBC sample—*M. tuberculosis*; MTBC Au-nanoprobe in the presence of a non-MTBC sample; and MTBC Au-nanoprobe in the presence of a non-complementary sample—non-related. After colour development a photo of the paper plate is captured and RGB image analysis is performed.

for the identification of DNA from MTBC. This novel integrated platform—*Gold on Paper*—holds great promise for a fast and cheap molecular diagnostics strategy for TB diagnostics at point-of-need. For the first time, a nanodiagnostics approach for the molecular characterisation of TB is combined with a simple paper based platform for use as a tool for the unequivocal identification of *Mycobacterium tuberculosis* in an easy to use lab-on-paper strategy. The integration of the *Gold on Paper* platform with the data analysis strategy on a mobile device may be proven as an effective simple approach to TB molecular screening in less than 2 h.

2 Materials and methods

2.1 *Gold on Paper* platform preparation

Starting from sheets of a cellulose substrate Whatman No. 1 Chromatography paper (Whatman International Ltd., Floram Park, NJ, USA), A5 standard format (210 × 148 mm) sheets were cut. This paper size fits directly into the manual feed tray of a commercial solid ink printer (Xerox ColorQube 8570, Xerox Corporation, Norwalk, CT, USA) designed to print a wax based ink, originating hydrophobic barriers.[13] Paper microplates were designed with a standard 384 plate format in Microsoft Office Visio® (Microsoft Corporation, US). All the measurements were taken from the "Microplate Dimensions Guide, Compendium of Greiner Bio-one microplates" (Greiner Bio-One GmbH, Frickenhausen, Germany). The printed pattern of a 384 microplate was placed on a hot plate (Heidolph MR Hei-Tec, Schwabach, Germany) at 140 °C for 2 min, allowing the wax to melt and spread vertically through the whole thickness of the paper, creating the desired hydrophobic pattern—see the ESI for production and characterisation.†

Each well was impregnated with 1 μL of a 0.12 M $MgCl_2$ solution (revelation agent) and allowed to dry at 25 °C for 10 min. The final microplates were stored at 25 °C, and wrapped in aluminium foil until use.

2.2 Scanning electron microscopy (SEM) analysis

Samples were mounted on aluminium stubs with carbon tape and coated with an 8 nm thick palladium-gold film in a Quorum Q150T ES sputtering system. The sample surface was observed in a Carl Zeiss AURIGA Crossbeam SEM-FIB workstation, using an accelerating voltage of 2 KeV with an aperture size of 30 microns.

2.3 Sample DNA preparation

A specific Polymerase Chain Reaction (PCR)-amplified 395 bp fragment of the *M. tuberculosis* RNA polymerase β-subunit (*rpo*B-GenBank accession no. L27989) gene suitable for detection of MTBC members was used as target for the Au-nanoprobe detection assay. PCR amplification was performed in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.2 mM $MgCl_2$, 200 mM of each dNTP, and 1 U of *Taq* DNA polymerase (Amersham Biosciences, GE Healthcare, Europe, GmbH), 10 pmol of each primer (P1 5′-GAG AAT TCG GTC GGC GAG CTG ATC C-3′; P2 5′-CGA AGC TTG ACC CGC GCG TAC ACC-3′)[3,25] involving 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 58 °C followed by 45 s extension at 72 °C. Amplification was observed by 1% Agarose gel electrophoresis and was further confirmed by direct sequencing using Big Dye v3.1 Terminator technology (STABVIDA, Almada, Portugal). DNA samples isolated from *M. tuberculosis* and non-MTBC Mycobacteria cultures were used as positive MTBC (complementary) and non-MTBC (non-complementary) samples, respectively. DNA from an unrelated organism was used as non-related. The non-MTBC sample is derived from a clinical isolate from *Mycobacterium kansasii*, whose sequence differs from that of the *M. tuberculosis rpo*B target region by a single nucleotide (see the ESI†).

2.4 Au-nanoprobe synthesis and characterisation

Gold nanoparticles, with an average diameter of ~14 nm, were synthesised by the citrate reduction method described by Lee and Meisel.[26] Briefly, 250 ml of 1 mM $HAuCl_4$ was heated while stirring, 25 ml of 28.8 nM sodium citrate was added, and the solution was refluxed for 15 min. Afterwards, the solution was left at room temperature to cool down. The gold nanoprobes were prepared by incubating the thiol-modified oligonucleotides with the AuNPs for 16 h. The solution was washed with 10 mM phosphate buffer (pH 8), and increasing salt concentration, in order to reduce non-specific binding between the thiol-modified oligonucleotides and the AuNPs. The solution was centrifuged, the resulting pellet resuspended in 10 mM phosphate buffer (pH 8), 0.1 M NaCl, and stored in the dark at 4 °C until further use.[27]

A comparative analysis of *rpo*B gene sequences from mycobacteria was performed through sequence alignment using Geneious v.4.7.6 software. Probe specificity was tested *in silico* using BLAST tools from GenBank. The MTBC probe 5′-thiol-GAT CGC CTC CAC GTC C-3′ (STABVIDA, Portugal) was then used to functionalise the AuNPs as previously described.[20,22] For target discrimination assessment in assay calibration, non-modified synthetic oligonucleotides were used, MTBC sequence 5′-GGA GAT TGG TTT TGA CGT TTA TGT GGA CGT GGA GGC GAT C-3′; and non-MTBC 5′-GGA GAT TGG TTT TGA CGT TTA TGT GGA TCG CCT CCA CGT C) positive (complementary) and negative (non-complementary), respectively. These calibration data were used to assess the probe specificity in the presence of the DNA sample.

2.5 Au-nanoprobe colorimetric assay

The 395 base pair (bp) PCR products were ethanol precipitated, resuspended in deionised water and used for the Au-nanoprobe assay (see also the ESI†). Each colorimetric assay was performed in a total volume of 30 μl, with Au-nanoprobes at a final concentration of 2.5 nM in 10 mM phosphate buffer (pH 8), 0.1 M NaCl and target DNA at a final concentration of 30 μg ml^{-1}, as previously described.[20,22] The assay involves the visual comparison of a "Blank" (without DNA), 10 mM phosphate buffer (pH 8), 0.1 M NaCl; "Probe", 10 mM phosphate buffer (pH 8); and the samples. After 10 min at 95 °C for target DNA denaturation, the mixtures were allowed to stand for 30 min at room temperature and $MgCl_2$ was added-[$MgCl_2$] = 0.02 M for the MTBC probe. After 30 min at room temperature, for colour development, the mixtures and the blank were assayed by UV/visible spectroscopy in a microplate reader (Tecan Infinite M200).

For assaying with the *Gold on Paper* platform, a total reaction mixture of 5 μL was used with 2.5 nM Au-nanoprobes in 10 mM phosphate buffer (pH 8), 0.1 M NaCl and target DNA at a final concentration of 30 μg ml^{-1}. After 10 min of denaturation at 95 °C, the mixtures were allowed to stand for 10 min at room temperature and spotted onto the well on the paper plate. After 45 min at room temperature for colour development, the paper plate was photographed with a mobile device and RGB analysis was performed.

2.6 Data acquisition and analysis

The colour pattern on the *Gold on Paper* was captured with an HTC Desire android smartphone with a 5 megapixel camera (2592 × 1944 pixels) with autofocus. Photos were taken with artificial white light without flash. Blank test spots with 10 mM phosphate buffer (pH 8), were used to normalise the data for light conditions. The digitalised data were then analysed without further manipulation with a free RGB analysis application (ColorPikr, WiseClue) and transmitted *via* a 3G network to a personal computer for image processing with ImageJ™. Each assay was repeated at least three times and on four different paper micro well plates.

A one-way ANOVA analysis, with Tukey's multiple comparison test, using GraphPad (San Diego, CA) was used to validate the results. The one-way analysis of variance was used to test for differences between two groups of data (Blank, MTBC sample, non-MTBC sample and non-related). Additional statistical analysis of the differences was carried out using Tukey's multiple comparison procedures.

3 Results and discussion

The *Gold on Paper* diagnostics strategy is depicted in Fig. 1. First, the Au-nanoprobes are incubated with the sample DNA for molecular recognition to take place. Then, the reaction mixture is spotted onto the paper microplate well for colour revelation. The colour change on the spots is then recorded by means of a camera on the generic mobile device and subsequently analysed *via* RGB.

To develop the *Gold on Paper* platform, we first optimised the Au-nanoprobe detection scheme for specific identification of DNA from *M. tuberculosis* (MTBC member). A specific Au-nanoprobe, targeting the *rpo*B *locus* shared by all MTBC members, was synthesised and optimised for use in the non-cross-linking detection method previously reported by our group.[20,22,28]

A minimum of three individual parallel hybridisation experiments targeting the 395 bp amplicons derived from the *rpo*B gene were performed. The Au-nanoprobe solutions exhibit a strong red colour derived from the localised SPR of the AuNPs. Upon salt induced aggregation, the presence of the complementary target sequence protects the Au-nanoprobes against aggregation and the solution remains red; the absence of a complementary DNA target sequence to that of the Au-nanoprobe results in extensive aggregation and the solution turns blue due to the red-shift of the SPR band. Based on the UV/visible spectra obtained after salt addition, the ratio between the absorbance at 526 nm (contribution of the non-aggregated fraction of Au-nanoprobes) and absorbance at 600 nm (contribution from the aggregated fraction of Au-nanoprobes) was calculated (Fig. 2A). A ratio of 1 may be considered as the point of equilibrium between non-aggregated and aggregated nanoprobes, hence the threshold to respectively discriminate between positive and negative samples. This way, it was possible to identify samples containing the target sequence.

Following calibration, the minimum amount of salt ($MgCl_2$) required for the aggregation of the Au-nanoprobes used in the calibration assay was impregnated in each well of the paper plate platform. The wax used for production of the paper microplate template retains the Au-nanoprobe solution and, because each well was impregnated with a probe specific concentration of salt, allows aggregation to occur. Paper-based microplates are well suited for work with small volumes of sample: 5 μL of solution is sufficient to uniformly fill a well in the 384 microplate standard format, while a plastic plate requires a minimum of 15 μL to fill the bottom of the well. This approach gives accurate results when detecting MTBC members, uses small volumes of sample (5 μL), allowing for a reduction of 6 times the total amount of probe and sample. After sample hybridisation with Au-nanoprobes, the 5 μL reaction volume was added to the paper microplate platform for colour development. After 45 min for solvent evaporation and colour development, a plate image was captured with a smartphone and the ratio between the red RGB channel and the blue RGB channel calculated—Fig. 2B and 2C (see also the ESI†).

The colorimetric data was then recorded with a smartphone and analysed directly on the device without further manipulation or was analysed after sending the information via 3G to a laptop for imaging processing (Fig. 2). The same samples were also evaluated in the traditional microplate reader setup. For all methodologies, the Au-nanoprobe system was capable of specific identification of *M. tuberculosis* DNA ($p < 0.0001$) with little variability between the methods and no loss of sensitivity and specificity when performing the colour analysis directly on the mobile device (see the ESI†).

The Au-nanoprobe assay is capable of discriminating the presence of the MTBC sample DNA down to a concentration of 10 μg mL^{-1} (*i.e.* 41 and 246 pmol of target for *Gold on Paper* and a microplate reader, respectively). The *Gold on Paper* platform shows a response to increasing sample DNA concentration similar to that of the microplate reader. However, the difference between positive and negative is less intense, although clearly statistically significant (see the ESI†).

Paper is a good medium for colour based spot tests as it provides a strong contrast and, due to the high surface-to-volume ratio, facilitates the accelerated evaporation of solutions and results in concentrated analytes. As can be seen in Fig. 2, the microplate signal is more intense than that of the Gold on paper platform. This can be easily explained since a smaller amount of Au-nanoprobes is being used. In fact, the 5 μL reaction volume (*versus* 30 μL on the microplate reader) allows for complete and uniform coverage of the entire test zone with sharp colour change, enabling direct visual detection. This slight decrease in signal is greatly compensated by the simplicity of the detection without loss of sensitivity, and does not require a microplate reader or UV/vis spectrophotometer.

Smartphone devices eliminate the need to transmit data to achieve the result, allowing a fast and reliable analysis. Effective

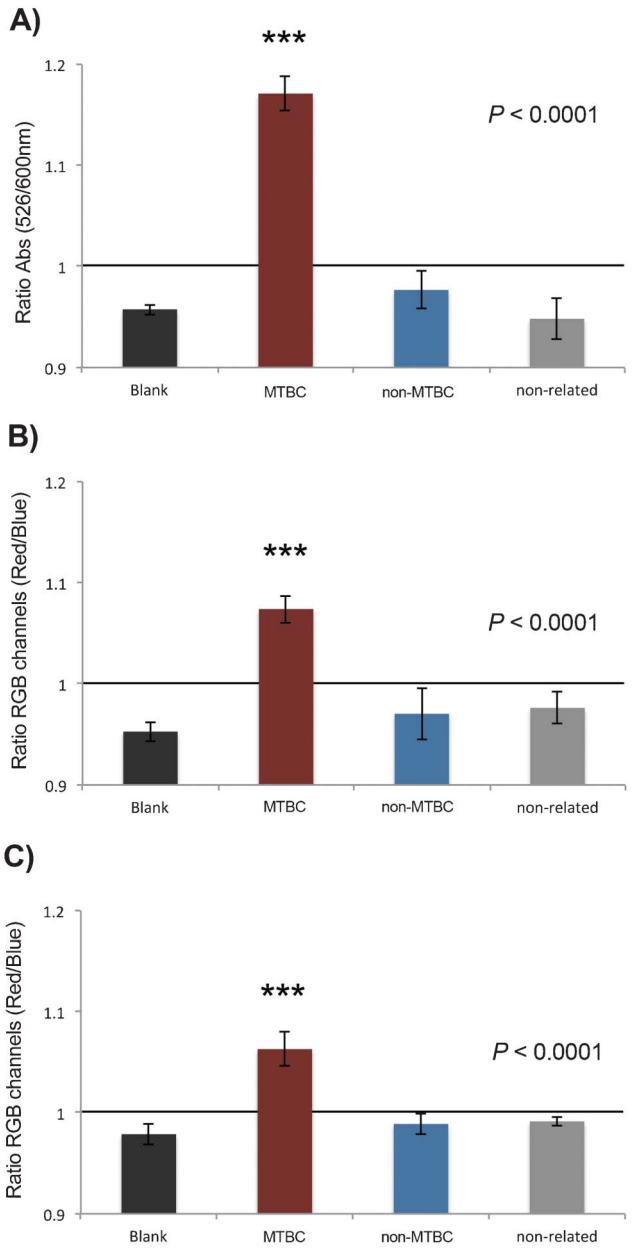

Fig. 2 Au-nanoprobe assay for MTBC detection: (A) MTBC standard assay performed in a microplate reader. Nanoprobe aggregation as measured by the ratio of aggregation (ratio of SPR intensity at 526 and 600 nm) for the assay mixtures—2.5 nM Au-nanoprobe, 10 mM phosphate buffer (pH 8), 0.1 M NaCl, and specific PCR amplified DNA at a final concentration of 30 μg ml^{-1} in a final volume of 30 μL. Sample detection, after 30 min incubation with [MgCl2] = 0.02 M; (B) MTBC assay performed in the paper microplate, Nanoprobe aggregation as measured by image RGB analysis. Ratio of aggregation calculated in ImageJ™ on a PC (ratio of average intensity of the red and blue channels) for the assay mixtures. (C) MTBC assay performed in the paper microplate, nanoprobe aggregation as measured by image RGB analysis. Ratio of aggregation calculated in the smartphone (ratio of average intensity of the red and blue channels) for the assay mixtures. Paper microplate assay performed with—2.5 nM Au-nanoprobe, 10 mM phosphate buffer (pH 8), 0.1 M NaCl, and sample DNA at a final concentration of 30 μg ml^{-1} in a final volume of 5 μL per test. Image captured after 45 min. The bars represent the average of three independent measurements and the error bars indicate standard deviation. The horizontal line represents the threshold of 1 considered for discrimination between positive and negative. Statistical analysis was performed using Prism 5 Graph Pad, using one-way ANOVA with Tukey's multiple comparison test; *** = $p < 0.0001$, n = 3.

TB diagnostics are achieved using *Gold on Paper* coupled to a mobile device and operational remote assessment is possible *via* RGB scrutiny.

After colour development three samples (paper microplate, MTBC positive and MTBC negative) were characterised *via* scanning electron microscopy (SEM) (Fig. 3). Results show a clear difference in the Au-nanoprobe dispersion in the presence of MTBC and non-MTBC target samples, the latter showing extensive Au-nanoprobe aggregation on the paper fibres. As shown above, these results show direct correlation with the colour captured in the digitalised images.

4 Conclusions

Serious efforts have been directed to provide robust, yet simple and portable molecular diagnostic platforms for use at peripheral laboratories and/or point-of-need. This is particularly relevant when assembling diagnostics platforms for worldwide epidemics with high numbers of affected people in less equipped regions of the globe, as in the case of tuberculosis. Here we show the potential of the *Gold on Paper* platform for TB molecular diagnostics that could be proven as a useful tool in the fight against TB. *Gold on Paper* is the working concept of integrating a paper microwell platform and a biomolecular detection scheme based on Au-nanoprobes. *Gold on Paper* was shown to be capable of efficiently detecting MTBC members directly and, by means of a smartphone device, analysing data on the spot while maintaining sensitivity and specificity. We have demonstrated that *Gold on Paper* detection is easy to perform without the need for expensive and complex laboratory set ups. Using *Gold on Paper*, it is possible to attain a positive identification of the pathogen within one hour, which *via* the use of a generic "smart" mobile device allows for complete analysis at a peripheral laboratory. We demonstrate that smartphones can be used as tools for data acquisition and handling in paper-based bioassays, eliminating the need to transmit data to get the results. Ultimately, we envisage a software package that collects and analyses the results on-site, and transmits the collected data to a centralised infrastructure. One issue hindering full implementation into remote locations may be the need for DNA sample preparation that now relies on PCR amplification, which is time consuming and costly. Nevertheless, efforts are being made to optimise this system *via* the removal of this limiting step. The limitation imposed by the DNA sample preparation is greatly overcome by the potential use of this methodology to identify and characterise the molecular signatures involved in antibiotic resistance.[22] This way, the additional level of information will be of most value to implement and/or adjust therapy. The whole process, including the PCR amplification step, occurs under 2h30, which is considerably faster than traditional methods.

Before *Gold on Paper* can be translated into clinics and to the point-of-need, extensive validation is still required. Also, the PCR step ought to be removed and/or modified without loss of

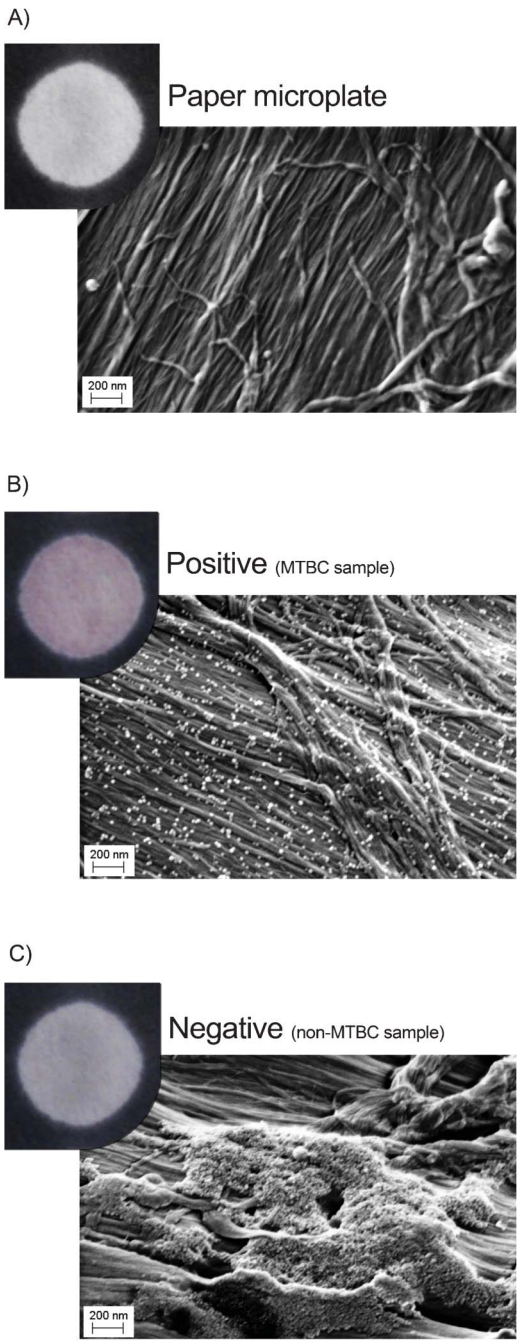

Fig. 3 *Gold on Paper* molecular diagnostics. Photographic captures of the detection on each well together with SEM images after the detection procedure. (A) Standard paper microplate well; (B) identification of a positive sample for *M. tuberculosis* complex (MTBC) showing the typical red colour on the spot. SEM image showing non-aggregated Au-nanoprobes; (C) Negative sample (non-MTBC DNA). SEM shows the extent of Au-nanoprobe aggregation on paper.

sensitivity, for example by including a paper microfluidics system for sample preparation.

Also, because we used paper as the platform for spotting and development of the aggregation assay, using SEM analysis it was possible to provide additional evidence of the differential aggregation events taking place in solution. For the first time, irrefutable evidence of the mechanism underlying the target-mediated aggregation of Au-nanoprobes *via* the non-cross-linking approach mechanism has been provided. In fact, the presence of the specific target prevents against salt induced aggregation and, as result, the solution retains the original colour; conversely, aggregation of the Au-nanoprobes upon salt addition occurs because no target is present to hybridise to the nanoprobe and hamper aggregation.

Based on *Gold on Paper* it is possible to perform the biorecognition and detection event and transmit digital information over existing communication channels, combined with geolocation metadata inserted into the captured digital images. This integrated diagnostics scheme can then forward the attained data to a centralised off-site server allowing for monitoring of TB in real-time that could be proven to be extremely useful in remote areas of the globe lacking resources. Future studies will be carried out to optimise the methodology towards validation, direct application to clinical samples, and to extend the range of applications to mutations associated with drug resistance.

Acknowledgements

This work was partially supported by the European Commission under project INVISIBLE (FP7 ERC grant n° 228144), and FCT/MEC: Strategic Projects PEst-C/CTM/LA0025/2011 and PEst-OE/SAU/UI0009/2011; Projects PTDC/EEA-ELC/099490/2008 (paper@), PTDC/SAU-BEB/098125/2008 (BloodFET), PTDC/CTM/NAN/109877/2009, PTDC/CVT/111634/2009. B. Veigas was supported by FCT/MEC (SFRH/BD/78970/2011).

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