One step visual detection of PCR products with gold nanoparticles and a nucleic acid lateral flow (NALF) device†

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Specific PCR products are detected with an antibody-free lateral-flow device by sandwiching them between reporter oligonucleotides covalently attached to gold nanoparticles (GNPs) and capture oligonucleotides covalently attached to a nitrocellulose chromatographic strip.

Lateral flow devices based on antibodies were first introduced in the 1980s and have subsequently become one of the most important products of the multibillion-dollar diagnostics industry. Their success has been based on a combination of low cost and simplicity that allows untrained personnel to undertake immunoassays in extra-laboratory environments such as a physician's office or hospital bedside without additional equipment. In their most simple embodiment these devices consist of a porous strip striped with a test line of antibodies or antigens, and fitted with a reagent pad impregnated with antibodies conjugated to a label that can be seen at low concentrations with the unaided eye.[1–3] Most often the porous strip is made of nitrocellulose and the label consists of GNPs. On insertion of this device into a sample, the liquid migrates along the chromatographic strip and solubilizes the labeled antibodies. Analyte antigens in the sample bind to the antibodies and their presence or absence is then indicated by the extent of colour development at the test line as the labeled antibodies bind to it. During the last few years these lateral flow devices have been adapted for nucleic acid detection,[4–6] but because they are still based on haptens and antibodies they are unnecessarily complicated and expensive. In a typical example, nucleic acids are amplified with haptenylated primers and detected by sandwiching them between antibodies conjugated to GNPs and a nitrocellulose membrane striped with adsorbed antibodies (Fig. 1A). In this communication we describe a nucleic acids lateral-flow (NALF) device that does not depend in any way on antibodies or haptens (Fig. 1B) and show how it can be used to detect unpurified PCR products with the unaided eye.

In order to avoid using GNPs conjugated to antibodies or an equivalent protein such as streptavidin, we conjugated reporter oligonucleotides directly to GNPs (for a full description of the methods see ESI†). Briefly high molecular weight mercaptodextrans were covalently attached to oligonucleotides and purified by covalent chromatography.[7] The purified dextrans were then

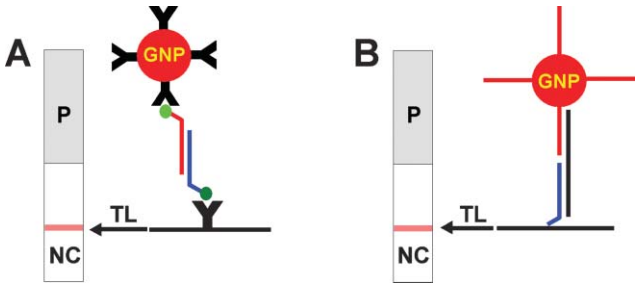

Fig. 1 Simple lateral flow devices consist of a strip of nitrocellulose (NC) striped with a test line (TL) of capture probes. When the device is inserted into a liquid sample, GNPs conjugated to reporter probes migrate along the nitrocellulose and through the test line in the direction of an absorbent pad (P). **A)** Section through test line of lateral flow device based on haptens (green circles) and antibodies. **B)** Section through test line of antibody-free lateral flow device.

conjugated to GNPs as described previously.[8] Simple lateral flow devices were constructed by striping (spraying) a sheet of plastic backed nitrocellulose with narrow lines of 5′-tailed oligonucleotides. Oligonucleotides tailed with poly-dT_{20} can be covalently attached to dried nitrocellulose by irradiating it with UV light. After covalent attachment of the capture oligonucleotides, the nitrocellulose was cut into strips and fitted with an absorbent pad. To demonstrate that these devices could detect nucleic acids we performed a series of sandwich assays for a 32 base target sequence (5′-GGGACTGACGATTCGGGTGATATCCAGAACGCAG-ACAAGCAGGCA) that had a 20 base 3′-terminal sequence complementary to the reporter oligonucleotides, and a 12 base 5′-terminal sequence complementary to the capture oligonucleotides. Fig. 2 shows images of strips acquired after performing a series of sandwich assays for different amounts of the target sequence in the range 0–10 pmol. The colour of the test line increased up to 2.5 pmol of target sequence and then levelled off and decreased due to the hook effect that occurred when the amount of target sequence exceeded the number of capture oligonucleotides anchored to the nitrocellulose; this is shown

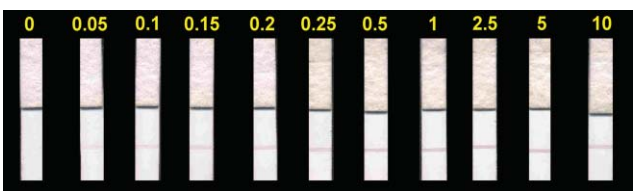

Fig. 2 Results of antibody-free nucleic acid lateral flow tests for a 32 base target sequence. Numbers show amounts of target sequence in pmol.

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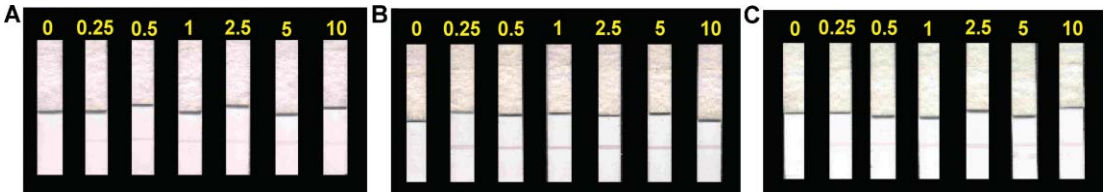

Fig. 3 Comparison between lateral flow tests developed with different sized GNPs. **A)** 40 nm; **B)** 80 nm; **C)** 150 nm. Numbers show amounts of 32 base target sequence in pmol.

graphically in the ESI†. There was no detectable colour development in the presence of 10 pmol of a non-complementary target sequence (5′-GACTCGGGGGATATCACTGATAACG-GTCAGGACGCAAAGGCACAG) and therefore the sensitivity (<50 femtomoles) was determined primarily by the amount of GNPs that could be seen with the unaided eye. The images in Fig. 2 show devices that were developed with 80 nm diameter GNPs, but we also performed assays with other diameters in the range 10–150 nm. Fig. 3 shows selected examples of how the amount of colour developed at the test line increased as the diameter of the GNPs increased from 10 to 80 nm (Figs. 3A and 3B) and then decreased as the diameter increased to 150 nm (Fig. 3C). A full set of images for all diameters studied is shown in the ESI†.

In all of these lateral flow tests the volume (50 μl) and O.D of the GNPs at 520 nm (1.0) was the same. In other words the total absorbing power of GNPs used to develop the devices was the same at all diameters studied. Fig. 4A shows the overlaid spectra of all the GNPs that were studied intersecting at 1.0 O.D. and Fig. 4B shows how the sensitivity of normal human colour vision varies with wavelength. As the diameter of the GNPs is increased there is a red-shift shift in the spectrum that eventually moves it into a region where the human eye is less sensitive. Fig. 4C shows how the number of GNPs in 50 μl of a 1.0 O.D. solution decreased as the diameter of the NPs increased; there were, for example, approximately 8 times as many particles in the 40 nm solution as there were in the 80 nm solution. In other words the extinction coefficient of the 80 nm GNPs (due to absorbance and scattering) is 8 times higher than the extinction coefficient of the 40 nm particles. The situation that obtains in chromatographic tests performed with GNPs having these diameters is shown in Fig. 5. On addition of the sample, target sequences hybridize to reporter oligonucleotides conjugated to the GNPs. Assuming the target sequences are evenly distributed each 80 nm particle is hybridized to 8 times as many target sequences as each 40 nm particle, and therefore the 80 nm particles have a higher probability of binding to capture oligonucleotides in the test line than 40 nm particles. If the extinction coefficient of the GNPs was the only factor responsible for the increase in sensitivity, it would go on increasing as the extinction of the particles increased, but as explained above the increase in diameter is also associated with a red shift in the absorbance spectrum that eventually moves it into a region where human vision is less sensitive. Therefore, instead of continuing to increase, the sensitivity levelled off at 80 nm, and then decreased as the GNPs became less visible to the human eye.

The results shown in Fig. 2 show that less than 50 fmol of single stranded target DNA can be detected with 80 nm GNPs, but the amount present in real samples is generally much less than this. The yield of DNA from a biopsy sample, for example, would typically be in the order of 3×10^5 copies (0.5 amol) of target sequence, which is well below the detection limit of our devices and of most other detection techniques. For this reason, an important requirement of devices like ours is that they can be interfaced with some form of amplification technique such as PCR. The results in Fig. 6 show the test lines of lateral flow devices that have been inserted into unpurified PCR mixtures (see ESI† for methods) at room temperature and the corresponding electrophoresis gel stained with ethidium bromide. It was possible to detect amplification products corresponding to 1000 template molecules (~0.002 amol) with the unaided eye. Although similar amounts of PCR product can be detected by staining electrophoresis gels with fluorescent dyes, this approach requires additional equipment and

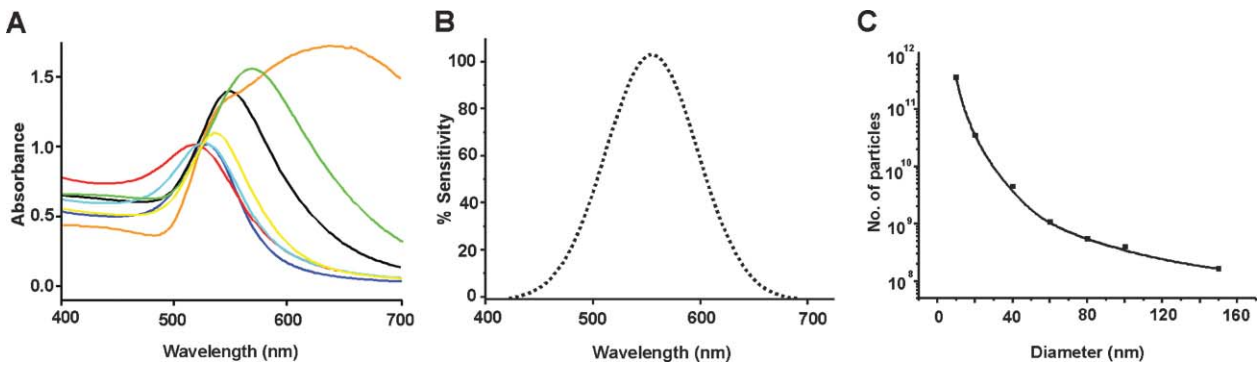

Fig. 4 **A)** Overlaid absorbance spectra of all GNPs used in the sandwich assays. Key: Red = 10 nm; Light blue = 20 nm; Dark blue = 40 nm; Yellow = 60 nm; Black = 80 nm; Green = 100 nm; Orange = 150 nm. **B)** Sensitivity of normal colour vision plotted against wavelength. **C)** Graph of experimental data and line of best fit showing how the number of GNPs in 50 μl of 1 O.D. suspension (the amount used in all chromatographic assays) decreases as the diameter of the GNPs increases.

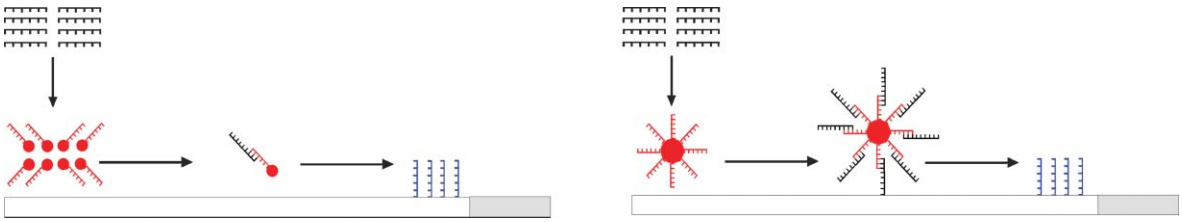

Fig. 5 Comparison between nucleic acids lateral flow tests performed with 40 nm GNPs (**left**) and 80 nm GNPs (**right**). Eight 40 nm particles must be captured by the test line to produce the same amount of colour development as one 80 nm particle, but the latter have a higher probability of being captured and therefore they generate a higher signal. This can be understood by thinking of the particles as spheres covered with Velcro hooks rolling along the strip and across a woollen test line orientated at right angles to the direction of their motion.

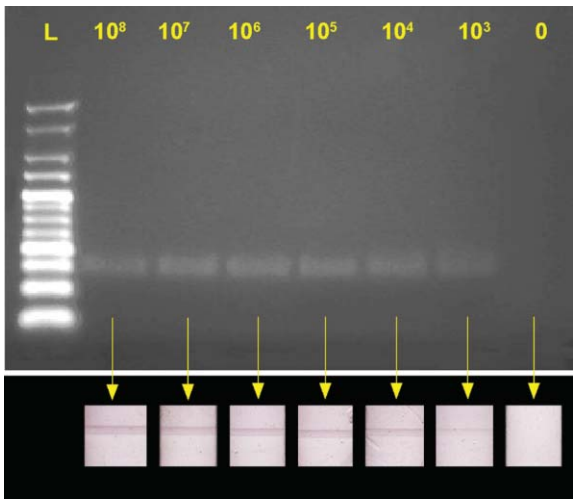

Fig. 6 Results of PCR assays. **A)** Agarose gel stained with ethidium bromide. **B)** Test lines of lateral flow devices developed with 80 nm GNPs. Numbers denote the amount of template sequence at the start of PCR.

the intervention of a skilled technician. The lateral flow devices also detect specific target sequences, whereas the stained gel only indicates that amplification products of a certain size have been generated and provides no other information about their identity.

In conclusion we have shown how unpurified PCR products can be detected with a lateral flow device that does not depend in any way on existing antibody hapten technology. To our knowledge this is the first time that such a device has been reported. A particular advantage of this approach is that it opens the way to detecting many nucleic acid sequences on the same test strip, an option that is not possible when immunochromatographic methods are used because of the limited number of antibody hapten combinations that are available for use with PCR. An unexpected outcome of this work was the discovery that the sensitivities of lateral flow devices can be increased by using 80 nm GNPs instead of the more commonly used 40 nm particles. Over a period of many years, the diagnostics industry has identified 40 nm as the optimum diameter because larger particles are believed to hinder biomolecular reactions by steric hindrance and be prone to aggregation.[9] As we have shown here, however, the use of larger particles is not only possible, but leads to an increase in sensitivity. Although we have used larger particles to increase the sensitivity of NALF devices, the same strategy should also enhance the sensitivity of lateral flow immunoassays. When GNPs are used the upper limit of the increase in sensitivity is imposed not by particle diameter *per se*, but by a red shift in the absorbance spectrum that accompanies the increase in size and moves it into a region where the human eye is less sensitive. In order to obtain further increases in sensitivity, therefore, it is necessary to use a label that retains the advantages of GNPs, but has a higher extinction coefficient in the region of the spectrum that is visible to the human eye. Silver NPs interact with light more efficiently than a particle of the same dimensions composed of any other known organic or inorganic chromophore, and have a plasmon resonance that can be tuned to any wavelength in the visible spectrum. Large silver NPs ($\sim$100 nm diameter) have absorbance maxima close to the wavelength at which the human eye is most sensitive,[10] and therefore their use as labels in lateral flow devices should lead to further increases in sensitivity.

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