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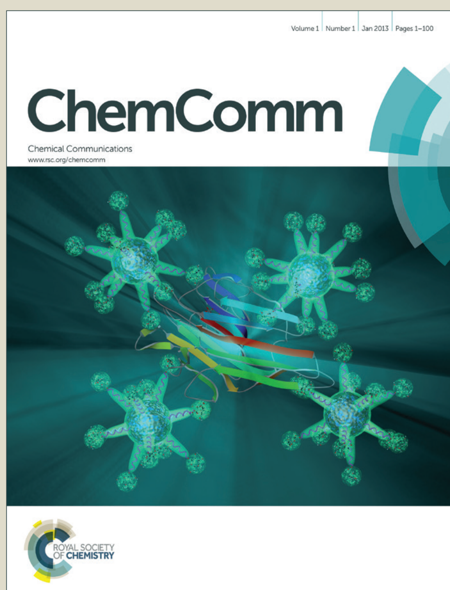

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COMMUNICATION

Aptamer-based biosensors for the rapid visual detection of flu viruses

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RNA aptamers showing affinity and specificity for different strains of human influenza virus were assembled onto gold nanoparticles that subsequently formed a gold nanoshell (AuNS) around the viral envelope. These shells could be visualised by transmission electron microscopy (TEM). Changes in size and structure of the AuNS coated virus can be used to detect the viruses. We show that sedimentation with a low cost centrifuge and visual determination can detect 3×10^8 viral particles.

Infection with the influenza virus can cause significant morbidity and mortality in both humans and commercially important livestock. In recent years, epizootics of one subtype of the highly pathogenic avian influenza virus A/H5N1 have led to the cull of millions of chickens and, according to the latest report from the World Health Organisation, 650 people in different countries are known to have been infected through contact with H5N1-carrying poultry, of whom 386 died.[1] Quick, simple and cheap tests to detect influenza virus can play a vital role in controlling infection spread as well as in guiding measures for appropriate avoidance, management and treatment strategies.

PCR and viral culture assays are highly sensitive and accurate methods for identification of viruses but they are costly and time-consuming requiring from several hours for the former to days for the latter.[2] A further limitation is that both these methods are laboratory -based and therefore are not suitable for field tests where special training and laboratory-based equipment is unavailable. Simple "dipstick" field tests from a number of commercial sources are available for human influenza virus detection using immunochromatography assays.[3] This type of test employs antibodies as the recognition molecules for the viruses. Comparisons between detection using PCR and viral cultures and the quick tests have been made in several publications and it has generally been found that the sensitivity and specificity in the former are traded for the convenience of the latter.[48] In this paper, we present work that is the foundation for building influenza's quick detection tools using nucleic acid aptamers. Aptamers have been proven to be comparable to antibodies as recognition molecules and have become widely used in analytical and diagnostic applications due to their advantages in terms of robustness and cost effectiveness.[9-11] Aptamers are typically selected through a process called SELEX (systematic evolution of ligands by exponential enrichment) and can have affinities comparable to antibodies [12; 13] but are smaller in size and more resilient in conditions that cause protein denaturation[9], and so well suited to field-testing.[10] In addition, aptamers are much cheaper to produce (typically 1,000 fold) once their sequence is known.[14] Furthermore, through incorporating counter selection steps into the SELEX process, it is possible to produce aptamers that are able to distinguish closely related molecules, for example the theophylline binding aptamer binds caffeine several orders of magnitude less tightly despite the two molecules only differing by a methyl group.[15] Similarly aptamers selected against an influenza A(H3N2) can discriminate between different strains of this subtype.[16]

Selection of aptamers against different viruses was reported in the 1990s, soon after the SELEX method had been published.[17] Both RNA and DNA aptamers were successfully selected with high binding affinities towards either the intact viruses or their component proteins. Initially it was hoped that aptamers could be potential anti-viral drugs and targets included HIV and Hepatitis B and C. [17-19] More recently, there have been several reports on selection of aptamers against the influenza virus in an effort to bring out new reagents for diagnostics and well as anti-viral drugs.[16; 20-23] Antigenic drift in influenza virus, particularly type A, is rapid as the virus can rapidly acquire mutations in their coat proteins: hemmagglutinin (HA) and neuraminidase (NA) [24; 25] resulting in a number of subtypes HxNy, where x = 1 to18 and y = 1 to 9 have been identified. For this reason, aptamers have been selected against HA, NA or even the whole virus particles.[16; 2-022; 26] The selection process for aptamers is rapid and also has ability to avoid selection

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against common epitopes that are found on older strains by using counter-selection. For example aptamers against an influenza (H3N2) strain Panama 2007/1999 can discriminate the older strains (Sydney 1997, Wuhan 1995, and Aichi 1968).[16]

Employing nanoparticles (NPs) as a label to facilitate detection is well established in analytical science.[27] NPs are well suited to point-of-care platforms and for near patient tests where simplicity is essential and, because of their intense absorbance at visible wavelengths, detection can be with the naked eye.[28] We show that aptamer modified gold nanoparticles (AuNPs) can be used to build simple point-of-care tests to detect the virus in a strain/serotype specific manner. These are not based on the well-established lateral flow assays [28; 29] but rather exploit changes in size and density when AuNPs form a shell that coats the viral envelope.

The strains chosen were based on two published aptamers that bind HA, the most abundant protein (75%) on the surface of influenza virus, of *B/Johannesburg/05/1999* (J1999V) and the (H3N2) influenza virus *A/Panama/2007/1999* (P2007V).[16; 21] The RNA aptamer for J1999V was selected against the purified haemagglutinin (HA) protein, of which the virus is known to have about 900 molecules on its surface.[21; 30-32] This aptamer is conjugated with 5nm AuNP's resulting in hundreds of AuNPs binding to the virus, whose diameter is around 100nm, forming a shell. We also used the same approach with the aptamer for P2007V, which was selected against the whole virus particle and where the HA protein was identified as the molecule to which the aptamer binds.[16] In order to use generic attachment chemistry, applicable to any DNA or RNA aptamer, a 5' biotin modified oligodeoxynucleotide was used as a universal linker attached to the gold surface through a streptavidin capping layer. Subsequently by producing the specific target recognition aptamer with a 3' extension complementary to the linker oligonucleotide, it could be readily assembled on the AuNPs. Binding of the NP-conjugated aptamer to the virus leads to the formation of a AuNS around the viral envelope. A schematic diagram of this is illustrated in Figure 1.

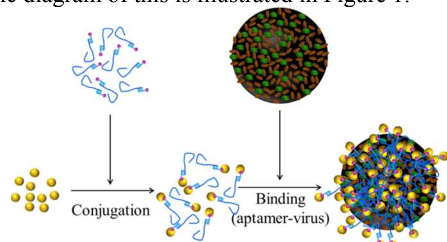

Figure 1. A schematic diagram of virus detection using aptamer-modified AuNPs. The streptavidin-capped AuNPs (gold spheres) are conjugated with the aptamer (blue lines) and binding to the virus (green/brown sphere) allows formation of a AuNS on the viral envelope.

The influenza viruses were inactivated by treatment with β-propiolactone[33] and their size and concentration were determined by NTA (nanoparticle tracking analysis).

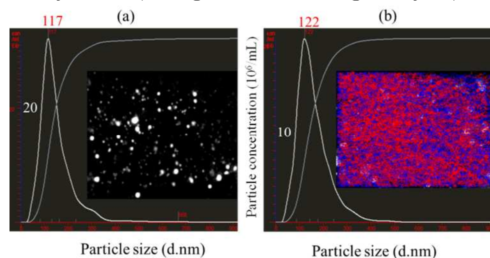

Figure 2. Determination of size and concentration of influenza virus particles using NTA. (a) J1999V and (b) P2007V. The inset of Figure 2(a) shows light scattering of the viral particles under Brownian motion whilst the inset of Figure 2(b) shows the tracking paths of individual virus particles.

Figure 2 shows the results of the NTA analysis which reveal that the mean particle diameter for J1999V was 117nm whilst P2007V had a mean particle diameter of 122nm. These values are in agreement with the literature.[34-36]

Changes in particle size upon binding of the aptamer-conjugated AuNPs to the viruses could be observed by DLS (dynamic light scattering). Detection based on changes in the size of scattering particles using DLS when the AuNPs bind to the virus have previously been reported by Driskell *et al.* using antibodies conjugated to AuNPs.[37] For a direct comparison with their work we used AuNPs of similar diameters conjugated with aptamers. DLS measurements of the aptamer-conjugated AuNPs with and without the viruses are shown in Supporting Information and confirm that the aptamer-AuNP conjugates can be used to detect the virus in the same way as antibodies. However, DLS is not a suitable technique for simple point-of-care tests.

The RNA aptamer selected against the purified HA protein of J1999V was reported to bind the protein with a K_d of 44 ± 6 nM using nitrocellulose membrane filtration with radioisotope labelling for detection.[21] Even though the RNA aptamer was selected against whole virus particles of the P200V, the binding affinity toward the purified HA protein was reported with a K_d value of 188pM using kinetic SPR (surface plasmon resonance).[16] We used ELONAs (enzyme-linked oligonucleotide assays) to investigate the affinities of these aptamers against the whole virus particles, not as purified proteins as ultimately they will used for direct detection without complex sample preparation. As shown in Figure 3, the aptamer for the J199V had a K_d of 28 ± 3 nM, which is similar to the reported K_d of 44 ± 6nM whilst the aptamer for P2007 showed a K_d of 1.6nM, almost an order of magnitude lower affinity compared to the reported value of 188pM. We consider this difference is understandable as the published affinity is to the purified HA whereas our affinity is for the whole virus.

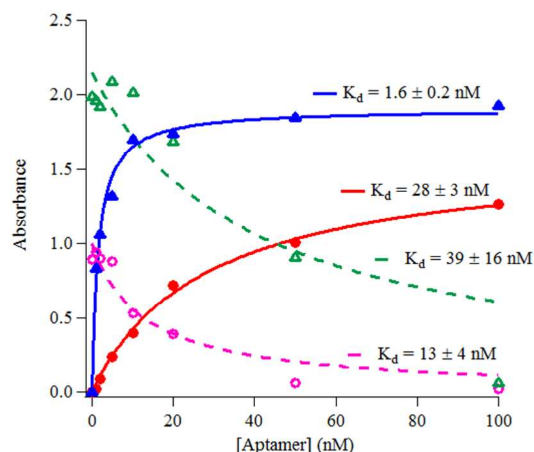

Figure 3. Direct (—) and competitive (– –) ELONAs. In the direct ELONA the binding of enzyme labelled aptamers to the virus is measured. In the competitive ELONA AuNP conjugated aptamers compete with enzyme labelled aptamers for binding to the virus. Details of the assays are in the Supplement.

In addition as the method involves using 5nm AuNPs as the detection moiety, the affinity of the AuNP-conjugated aptamer was also investigated to assess the effect of its attachment to the AuNPs on the affinity. The results shown in Figure 3 suggest there is no significant difference in binding affinity of the aptamer against whole virus J1999V particles irrespective of whether it is unconjugated or bound to the 5nm AuNPs. However, the P2007 aptamer lost an order of magnitude in

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affinity upon conjugation to 5nm AuNPs. This affinity is however still in the low nM region comparable to that of the aptamer for J1999V.

A detection method for field testing is ideally based on a visible endpoint. To this end, we used the well known observation that AuNPs with different sizes can be separated by centrifugation.[38; 39] As well as allowing the formation of a more compact AuNS on the viral envelope, using small AuNPs (5nm) will offer better separation between the AuNS.virus complexes and the unbound AuNPs. Confirmation of the specificity of the 5nm AuNPs modified with the aptamer to J1999V is shown by the TEM images in Figure 4(a) compared with the control where the virus is treated with AuNPs carrying a random RNA sequence of the same length in Figure 4(b).

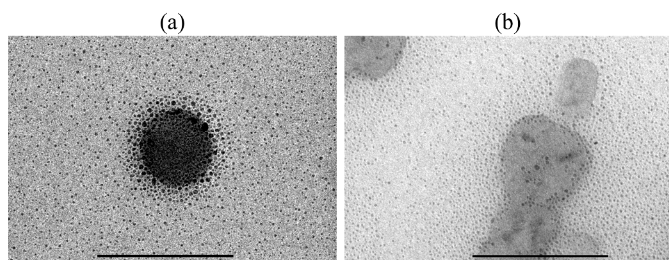

Figure 4. TEM images. (a) J1999V was coated with the specific aptamer assembled on AuNPs. (b) J1999V with a random RNA sequence of the same length assembled on AuNPs. The scale bar is 200nm.

Figure 4(a) shows formation of a dense AuNS on the viral envelope and this is consistent with the aptamer binding to the HA protein, which comprises 75% of the viral surface. As expected and confirmed by the image, there is a large difference in size between the unbound AuNPs and the AuNP-virus particles and that facilitates their separation and subsequent detection.

The sedimentation coefficient, s, of spherical particles suspended in a fluid can be calculated using Equation 1, derived from the Svedberg and Stokes Einstein equations[40; 41]

$$s = (d_p)^2 \frac{(\rho_p - \rho_b)}{18\eta_b} \qquad (Equation\ 1)$$

where d_p is the particle diameter, ρ_p is the density of the particle, ρ_b is density of the fluid and η_b is viscosity of the fluid.

The AuNS-virus is a complex particle consisting of 3 parts: the virus core, the 'joining layer' (streptavidin, linker, aptamer) and the AuNS shown schematically in Figure 5. The dimensions of the virus and the AuNS-virus were based on the NTA results (Figure 2) and TEM (Figure 4) data.

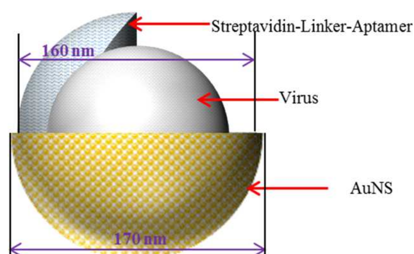

Figure 5. Schematic of the AuNP coated virus.

Influenza A and B viruses are reported to have densities of around 1.1×10^3 kg.m^{-3}.[36] In calculations using Equation 1, we treated the virus plus the streptavidin-Linker-Aptamer that made up a 160 nm sphere as having the same density as the virus and the AuNS having a density of 14.74×10^3 kg.m^{-3}, assuming 75% of the surface is covered by the 5mm AuNPs. This yields a density of 3.37×10^3 kg.m^{-3} for the AuNS-virus complex. Assumptions of 50%, 25% and 10% surface coverage by the 5nm AuNPs result in a AuNS density of 10.16×10^3 kg.m^{-3}, 5.58×10^3 kg.m^{-3} and 2.83×10^3 kg.m^{-3} and a density of 2.61×10^3 kg.m^{-3}, 1.84×10^3 kg.m^{-3} and 1.39×10^3 kg.m^{-3} for the coated virus, respectively. Influenza virus has around 900 copies of HA on each virus particle[21; 32] and with a 1:1 binding of 5nm AuNP to HA this yields a density of 1.50×10^3 kg.m^{-3} for the coated virus, corresponding to a 14% of surface coverage using the model in Figure 5. These values were used to calculate the sedimentation coefficient, s, from Equation 1. Once the sedimentation coefficient was known, the sedimentation velocity of the particle can be calculated using Equation 2[40; 41]

$$u = s\,(\omega^2 r) \qquad (Equation\ 2)$$

where u is the particle sedimentation velocity and $(\omega^2 r)$ is centrifugal field. Calculations using Equation 2 for the AuNS-virus particle with a centrifugation field of 2,000×*g* and assuming 75% surface coverage gave a sedimentation velocity of 5.7×10^{-5} m.s^{-1}, *i.e.* it requires 1 minute for the AuNS-virus complex particle to sediment a distance of 0.5cm. Calculations for the 50%, 25% and 10% surface coverage result in a time of 1.5, 3 and 6.5 minutes for the same sedimentation distance whilst the virus covered with 900 of 5nm AuNPs is 5 minutes. For comparison, it would require 167 minutes for the 5nm AuNPs to sediment the same distance. Using a portable Technico mini-centrifuge as shown in Figure 6 with a 10 minute centrifugation time gave particle sedimentation in samples containing the virus whilst no sedimentation was observed in samples without the virus. Using a domestic handheld electric whisk instead of a centrifuge yielded a similar result.

Figure 6. Visible observation of the virus-containing samples on the right side (3×10^9 viral particles) sedimented with a Technico mini-centrifuge whilst no visual sedimentation was observed for the no-virus samples.

In addition, experiments with simulated nasal mucus that contained 0.7mg/mL of total proteins of which 0.35mg/mL was mucin[42] were also conducted to assess the feasibility of the method in complex backgrounds. Using this approach we were able to detect 3×10^8 virus particles in both buffer and stimulated nasal mucus with the naked eye. This corresponds to

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the upper end of a typical viral load in 1mL of human respiratory specimens.[43]

This method of virus detection is as simple and quick as the current available dipstick tests that use antibodies on lateral flow formats. This gravity-based method could also be applied to antibodies as the recognition molecules instead of aptamers. Compared to antibodies, aptamers however are more robust in conjugation, allowing site specific biotinylation that results in single-point immobilisation on the AuNPs and they cost only 1/1000th that of the antibody on a per molecule basis. In addition, speed of generation of aptamers is generally quicker than monoclonal antibodies and with the use of counter selection it is possible to produce aptamers that are highly specific to even different strains of the same virus subtype.

Conclusions

We show that aptamers conjugated with small AuNPs (5 nm) through a universal oligonucleotide linker can bind to influenza virus particles. Formation of a AuNS around the viral envelope allows a simple visual detection of the viruses, suitable for field testing when the AuNP coated virus particles are separated using a low cost centrifuge or a domestic electric whisk. The generic nature of this approach means it could be extended to the detection of other viruses where there are specific aptamers that bind to them.[44]

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