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Comparative investigations for adenovirus recognition and quantification: Plastic or natural antibodies?

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ABSTRACT

Comparative and comprehensive investigations for adenovirus recognition and detection were conducted using plastic and natural antibodies to compare three different strategies. The implementation of molecularly imprinted polymer (MIP) technology for specific and sensitive recognition of viruses with the combination of biosensors was reported. Plastic antibodies (MIPs nanoparticles) were produced for adenovirus by employing a novel solid phase synthesis method. MIP receptors were then characterised using dynamic light scattering (DLS) and transmission electron microscopy (TEM) techniques prior to immobilisation on a surface plasmon resonance (SPR) sensor as affinity receptor for adenovirus detection. Two different templates were also imprinted as control MIPs (vancomycin-MIP and MS2-MIP). The specific recognition of adenovirus was investigated in the concentration range of 0.01–20 pM and the limit of detection was achieved as 0.02 pM. As an alternative to MIP receptors, direct and sandwich assays were developed for adenovirus quantification using natural antibodies. The detection limit of direct and sandwich assays were found as 0.3 pM and 0.008 pM, respectively. The kinetic data analyses were performed for three different adenovirus recognition methods and cross-reactivity studies were also conducted using MS2 phage as control virus and an excellent specificity was achieved with all assays types. This work confirmed the suitability of the MIPs SPR sensor for the detection of viruses.

1. Introduction

Although the standard of the world's drinking water has improved in recent years, there were still an estimated 748 million people worldwide, according to the World Health Organisation (WHO) in 2012 (around 9% of the world's population), without access to sanitary drinking water. Of these an estimated 173 million people depend solely on surface water (such as from streams, rivers, wetlands and lakes) for their day-to-day drinking water needs. Hence, accessibility of clean, pathogen-free water supplies makes a huge impact on the reduction of deaths and the increase of general public well-being (Cosgrove et al., 2014). To achieve this, biological contamination including pathogenic microorganisms must be eliminated or reduced to safe levels from drinking water supplies. Current methods used to disinfect water supplies include filtration, UV irradiation, addition of chemical oxidants such as chlorine and/or thermal treatment. However, viruses due to their small size, lack of metabolism, protective protein coat and the protection supplied by associated organic matter can be more difficult to inactivate from water supplies, making them an area of special attention.

The enterically transmitted viruses are the major causes of waterborne diseases in humans. These viruses are infectious in low doses (generally only a few virion particles are required for infection), stable in the environment for extended periods of time and difficult to study. Viral detection in water requires specialised analytical methods and experienced technicians. One example of a virus which is both pathogenic to humans and transmitted via contaminated water is adenovirus, responsible for pneumonia as well as ocular, respiratory and enteric infections (Sinclair et al., 2009). Diagnosing infections, however, is not simple as they produce little symptoms indicative of an infection until later in progression. Conventional methods of diagnosing viruses such as the polymerase chain reaction (PCR) have limitations due to the PCR's susceptibility to hybridisation errors and high equipment maintenance needs (Chuang et al., 2012). High-throughput analyses such as enzyme-linked immunosorbent assays (ELISAs) also carry their caveats, for example the ELISA's undesirably long operating time of 3–5 h and the possibility of non-specific signals generated by cross-reactivity of the secondary antibody (Lei et al., 2013).

Efforts have been made to develop novel diagnosis methods for

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viruses, using techniques such as atomic force microscopy (Meillan et al., 2014), interferometry (Ymeti et al., 2007) and electrochemistry (Lee et al., 2013; Wei et al., 2013), or by implementing components such as carbon nanotubes (Dias et al., 2013; Tam et al., 2009), silicon nanowires (Svendsen et al., 2011), liquid crystals (Han et al., 2014), microcavities (Dantham et al., 2012) and microfluidics (Connelly et al., 2012; Matatagui et al., 2013) into sensor systems. Many of these studies, as well as others in the field, have used antibodies as the recognition material for virus detection exploiting the high specificity between an antibody and its analyte as a detection system (Bianco et al., 2013; Altintas et al., 2011). They also demonstrate high affinity for their target and have a proven, successful track record of implementation into reliable, effective and portable biosensor systems, such as in pregnancy tests (Hu et al., 2014). However, their maintenance can be difficult, they can be very expensive (Tothill, 2009), are prone to oxidative/microbial degradation and are unusable in harsh environments such as strong acidic/basic conditions or organic solvents (Altintas et al., 2015a).

An alternative to antibodies circumventing these factors is the use of molecularly imprinted polymers (MIPs). These receptors are designed and synthesised to function as artificial antibodies to recognise and bind specifically to a target (Volkert and Haes, 2014). They carry the advantages of being more cost-effective, as well as having greater stability in harsh conditions than antibodies. They can also be used against molecules that adversely affect immune responses such as toxins or immunosuppressants. However, it is more difficult to imprint larger targets such as viruses, (particularly because their three-dimensional protein structures are flexible and can fluctuate under minimal influence) and MIP technology is still without a standard manufacturing procedure (Poma et al., 2010). Because of this, MIPs generally have lower specificity and sensitivity than antibodies (Tothill, 2009). This is not to say that MIPs for viruses have not been successfully produced (Bolisay et al., 2007; Sankarakumar, 2013). Like antibodies, MIPs can be applied onto sensor systems (Ge et al., 2013; PauláGleeson, 2013), and MIP-based sensors are growing in interest to researchers because of their potential in bringing down the cost of sensors.

In this study, we explored the application of adenovirus-targeted MIPs onto a prism-based SPR biosensor system, and observed the performance of the MIPs in comparison to a MIP targeted to bacteriophage MS2 (a model virus used commonly in research), vancomycin-targeted MIPs (used as a control) as well as an antibody against the adenovirus serotype 5. The adenovirus MIPs were also tested for their ability to bind to bacteriophage MS2, assessing their specificity. The MIPs were produced by an adapted chemical method (Hoshino et al., 2008) using glass beads as a solid support for immobilising the target viruses while the polymerisation took place.

2. Materials and methods

2.1. Reagents and Chemicals

N-isopropylacrylamide (NIPAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), acrylic acid (AAc), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), *N*,*N*′-methylenebisacrylamide (BIS), *N*-tert-butylacrylamide (TBAm), phosphate buffered saline (PBS) tablets, tris(hydroxymethyl)aminomethane (TRIS), glutaraldehyde (GA), 3-aminopropyltrimethyloxysilane (APTMS), 11-mercaptoundecanoic acid (MUDA), TWEEN® 20 (polyoxyethylenesorbitan monolaurate), cysteamine, methanol, ethanol, toluene, and acetone were purchased from Sigma Aldrich (UK). *N*-(3-aminopropyl) methacrylamide hydrochloride (APM) was purchased from Polysciences Inc. (Pennsylvania, USA). Double-distilled ultrapure water produced by a Millipore Direct-Q® 3 UV (Millipore; Molsheim, France) was used for analysis. Bacteriophage MS2 and inactivated adenovirus serotype 5 viruses were prepared by Public Health England (Porton, Wiltshire, UK). The anti-adenovirus serotype 5 antibody was obtained from Abcam (Cambridge, UK). All chemicals and solvents were analytical or HPLC grade and were used without further purification.

2.2. Apparatus and equipment

Glass beads were obtained from Sigma Aldrich (UK). Whatman® filter papers were purchased from Camlab (Cambridge, UK). A Retsch AS200 shaker (Retsch Inc.; USA) was used to agitate ceramic beads with the glass beads. A Zetasizer Nano (Nano-S) from Malvern Instruments Ltd. (Malvern, UK) was used to determine the size of the MIP nanoparticles by dynamic light scattering (DLS). A Scanvac Coolsafe freeze-dryer (LaboGene, Denmark) was used to determine the yield of MIP nanoparticles. Gold-coated sensor chips (SIA Kit Au, BIAcore) were purchased from GE Healthcare Biosciences AB, Sweden. A BIAcore 3000 SPR system (BIAcore, Sweden), as well as an SPR-4 sensor system (Sierra Sensors, Germany) was used to monitor the binding interactions (association and dissociation) between the MIP nanoparticles and their templates. A Grant sonicator (Patterson Scientific, Luton, United Kingdom) was used to dissolve solutions thoroughly as required. A transmission electron microscope (Philips CM20, Philips Research) was used to depict the adenovirus and the synthesised adenovirus MIP receptors.

2.3. Preparation of the virus culture media

2.3.1. Preparation, concentration and purification of adenovirus

T175 flasks of Human Embryonic Kidney (HEK) 293 cells were grown to 50–70% confluency in DMEM-10 [Dulbecco's Modified Eagle's Media (DMEM, Gibco)+10% Foetal Bovine Serum (FBS, Sigma)+1% Non-Essential Amino Acids (NEAA, Sigma)] and inoculated with 5×10^7–5×10^8 pfu of adenovirus 5 stock culture (National collection of pathogenic viruses, Public Health England, Porton Down) (diluted in DMEM-10) to give a final multiplicity of infection (MOI) of 10–20 pfu/HEK cell. The flasks were incubated at 37 ± 2 °C in a 5% CO_2 humidified environment for 60 min after which 10 mL of DMEM-10 was added to each flask. Flasks were further incubated at 37 ± 2 °C in a 5% CO_2 humidified environment for 2–3 more days checking the cytopathic effect daily until full cytopathic effect had been reached with little or no adherence of the cell layer. The virus was harvested by removing the still adherent cells from the flask's surface using a cell scraper and centrifuging the harvested material at 3000 g (5000 rpm) for 15 min. All 2 mL of supernatant was decanted into a sterile flask, with the remaining 2 mL of supernatant being used to re-suspend the cell pellet. The re-suspension was rapidly frozen in a bath of dry ice with a small amount of isopropanol, rapidly defrosted in a 37 °C water bath, and vortexed for three successive sets of five minutes in order to lyse the remaining cells and release virus particles into the media. The media was then centrifuged at 3000 g for 15 min, with the resulting supernatant decanted into the sterile flask containing the initial supernatant, and the cell pellet was discarded (Pottage et al., 2010). The supernatant was $CsCl_2$ (Sigma) ultracentrifuged using 0.51 g $CsCl_2$/g of solution (to make the density of the solution 1.34 ± 0.001) for at least 24 h at $301{,}000\times g$ (50,000 rpm), and the resulting band of concentrated virus was removed using a syringe and dialysed into PBS twice for 24 h using a Slide-A-Lyser dialysis cassette (20,000 MWCO). The TEM image of adenovirus is given in Fig. 1b.

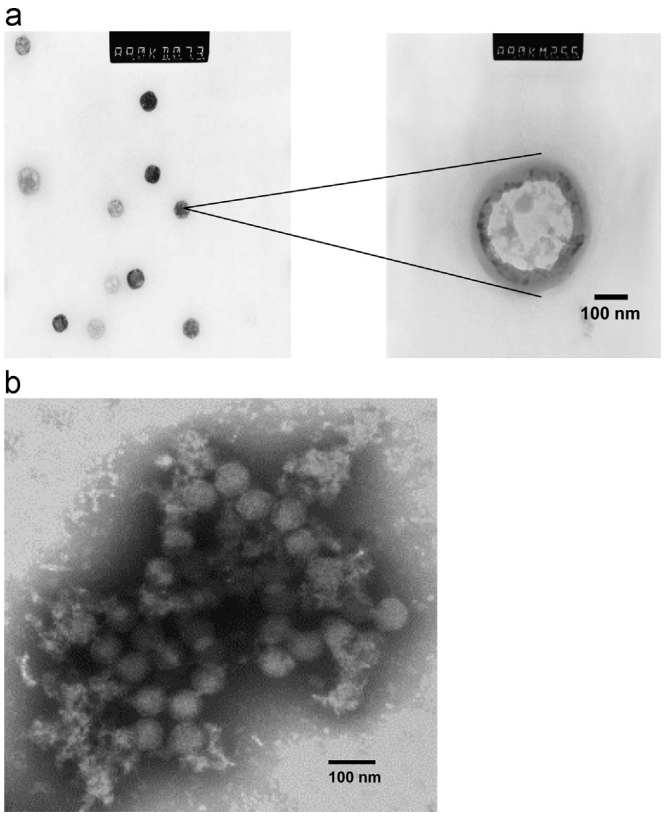


Fig. 1. TEM images of the produced adenovirus-MIP with the size around 260 nm (a) and adenovirus as small spheres (b).

2.3.2. Adenovirus plaque assay

HEK 293 cells were seeded into 6-well plates to form a confluent monolayer after overnight incubation at 37 °C in a 5% CO_2 environment. The media was gently removed from the wells and 800 μL of fresh DMEM-10 was added carefully before returning the plates to the incubator for 2 h. Approximately 30 min before the plates were ready to be used; DMEM-10 was used to perform a ten-fold serial dilution of the test adenovirus. After the plates were removed from the incubator, 200 μL of each dilution was added to their respectively-labelled wells. The plates were then rocked gently to disperse each dilution evenly before being placed back into the incubator. This day was recorded as day 0 of the assay. On day 1, the virus was removed from the plates using a 5 mL pipette and a mixture of 12 mL DMEM-10 held at 37 °C and 1.15 mL molten 4% agarose (Type II, Sigma) held at 65 °C was prepared and 2 mL of this mixture was added to each well of the plate. This was allowed to set before returning the plate to the incubator. On day 4/5 and 7/8, additional agarose plugs were added to the wells using the method mentioned above. On day 8/9, 200 μL of neutral red stain was added to each well, and the plates were returned to the incubator for 24 h (Pottage et al., 2010). The plaques were then counted and used to determine the plaque forming units per millilitre of the original virus suspension.

2.4. Preparation of derivatised glass beads

2.4.1. Glass beads preparation

A Retsch AS200 shaker was used to vibrate a bulk of glass beads (approximately 300 mL of beads between 45 and 90 μm in diameter) for 5 h with abrasive ceramic beads. To activate the glass beads, they were boiled in an excess of 1 M NaOH for 1 min. After this, they were washed six times with an excess of double-distilled water at 60 °C, followed by a single wash with an excess of acetone at room temperature, then left to dry completely in an oven at 80 °C (> 2 h). A short-linker silane coat was added to the glass beads by incubating them in a 2% v/v solution of APTMS in anhydrous toluene overnight.

2.4.2. Glutaraldehyde coupling

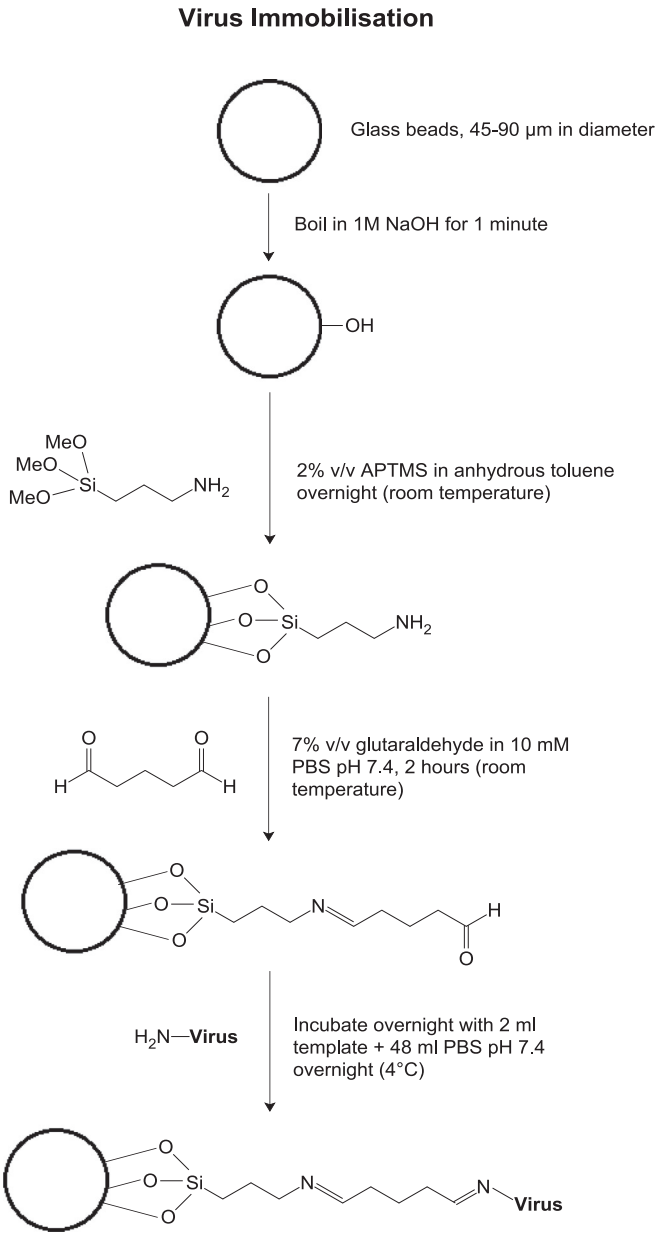
60 g of silanized glass beads were washed thoroughly with acetone under vacuum and left to dry in the ceramic filter funnel for approximately 30 min. The beads were then incubated in 7% v/v glutaraldehyde solution in PBS (pH 7.4, filtered using 0.2 μm filter paper and degassed for 10 min) for 2 h, before rinsing with double-distilled water under vacuum. 2 mL of the template (virus or vancomycin) was immobilised by incubating the glass beads in a 50 mL solution of PBS overnight at 4 °C. Finally, the beads were rinsed with double-distilled water under vacuum before drying again. The concentration of virus added to the glass beads was too low to bind to all the glutaraldehyde; therefore, unbound, reactive glutaraldehyde needed to be blocked with ethanolamine, otherwise it is possible for glutaraldehyde groups to couple with each other, which has an adverse effect on nanoparticle formation. 50 mL ethanolamine (0.1 mM) in PBS (pH 7.4) was used to incubate beads at room temperature for 15 min, after which the beads were washed with double-distilled water, dried and kept in the fridge. Scheme 1 visualises the process of preparing the glass beads for solid-phase MIP synthesis.

2.5. Plastic antibody synthesis and characterisation

The synthesis of MIP receptors for adenovirus, bacteriophage MS2 and vancomycin was adapted from a protocol by Hoshino et al. (2008). MIPs were produced using the glass beads as a solid-phase support for the template (Abdin et al., 2015; Altintas et al., 2015b). In 98 mL of double-distilled water, the following components were added; 39 g *N*-isopropylacrylamide, 2.2 μL acrylic acid, 2 mg *N*,*N*′-methylenebisacrylamide, 33 mg *N*-tert-butylacrylamide, dissolved in 2 mL ethanol, 5.4 mg *N*-(3-aminopropyl) methacrylamide hydrochloride. The solution was sonicated, then degassed for 10 min under vacuum and purged with nitrogen for 30 min. All of these procedures were carried out in a two-way flask. 180 mg APS was dissolved in 3 mL double-distilled water, and 90 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was added to this mixture before adding 824 μL of this mixture to the previous monomer mixture. The mixture was polymerised under nitrogen atmosphere at room temperature for 2 h. The mixture was washed six times with double-distilled water at 15 °C, and the MIP nanoparticles were then eluted by passing three fractions of double-distilled water through the column at 60 °C (each three fractions constitutes one batch of MIP nanoparticles). The schematic illustration of MIP receptor synthesis for adenovirus is given in Supplementary document (Fig. S1) and the components for MIP synthesis are also represented with their structures in Table 1. The harvested MIP receptors were sonicated for 30 s and a 1 mL sample was analysed by DLS using a Zetasizer Nano (Nano-S), to obtain information on their size and consistency.

2.6. Determination of plastic antibody yield by freeze-drying

The weight of a 50 mL plastic vial was weighed accurately on a microbalance and recorded. Following this, 10 mL of MIP solution was placed into the vial and kept in the freezer until fully frozen. The vial was inserted into a Büchner flask, which was stoppered, and had two sequential 0.2 μm filters attached to the hose barb. The flask was placed into a freeze-dryer set to approximately −55 °C for around 48 h. After the solvent had dried out, the vial was re-weighed, and the weight increase was recorded.



Scheme 1. Schematic illustration of glass bead preparation with template for solid-phase MIP synthesis.

2.7. MIP concentration by evaporation

Before the MIPs were to be used in any SPR experiments, a 10 mL sample of MIP solution was taken from a batch and put in a small glass vial. This was clamped in a water bath kept at 55 °C, and bubbled with nitrogen gas until approximately 1 mL of solution was left. This 1 mL solution was stored at 4 °C until use.

2.8. Adenovirus sensor assays

2.8.1. Plastic antibody-based assays

Unless a brand new chip was about to be used, the gold chips were washed with distilled water, placed in a glass container and covered with piranha solution (3:1 H_2SO_4:H_2O_2) for 15 min under the fume hood. To stop the reaction, the piranha was diluted using distilled water, after which the chips were placed in a Petri dish and covered with a 50 mM solution of 11-mercaptoundecanoic acid (MUDA) in ethanol overnight, sealing the dish and covering it with opaque foil before incubating. The chips were then washed with ethanol for 2 min, then washed again with distilled water and dried carefully with nitrogen gas before attaching them to the plastic covers. After this, they were stored at 4 °C until use. Each running buffer prepared for SPR experiments was filtered using 0.2 μm filter paper and degassed before use. The flow rate of injection was kept at 10 μL min^{-1} for the Biacore 3000 and the dissociation time was maintained at 180 s between one injection finishing and the next starting. The MIPs were immobilised on the sensor chips after activating the surface with a three-minute injection of EDC/NHS solution (0.4 M EDC, 0.1 M NHS), and any remaining binding sites were blocked with a three-minute injection of 1 M ethanolamine. Virus samples were then prepared and affinity-based virus binding assays for the MIPs were carried out in the concentration range of 0.01–20 pM. Cross-reactivity studies were also conducted using MS2 phage to check the specificity of the adenovirus MIP against its own target. Moreover, vancomycin-MIP was imprinted as completely different MIP to be used as a control MIP receptor and screened against the adenovirus in addition to other virus-MIP control (MS2-MIP).

2.8.2. Natural antibody-based adenovirus sensor assays

Adenovirus antibody was covalently immobilised onto MUDA coated sensors chips using the same strategy with plastic antibodies as described before (Altintas et al., 2011, 2012a, 2012b). A 50 μg mL^{-1} of antibody was prepared within sodium acetate buffer (pH: 4.5) after the optimisation studies to determine the most convenient antibody concentration, pH and running buffer conditions. Adenovirus samples were then prepared in PBS-T in the concentration range of 0.15–20 pM and 0.004–0.5 pM for direct and sandwich assays, respectively. For sandwich assays, a 5 μg mL^{-1} concentration of adenovirus detector antibody was used based on the preliminary tests. Cross-reactivity test was also conducted in the concentration range of 0.15–20 pM using MS2 phage samples.

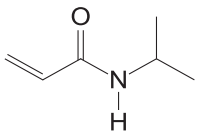
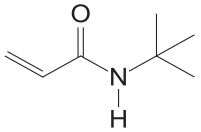
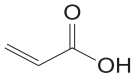
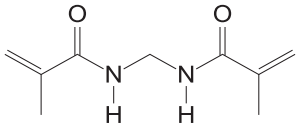
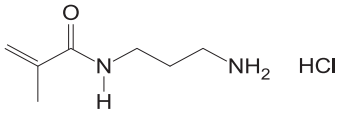
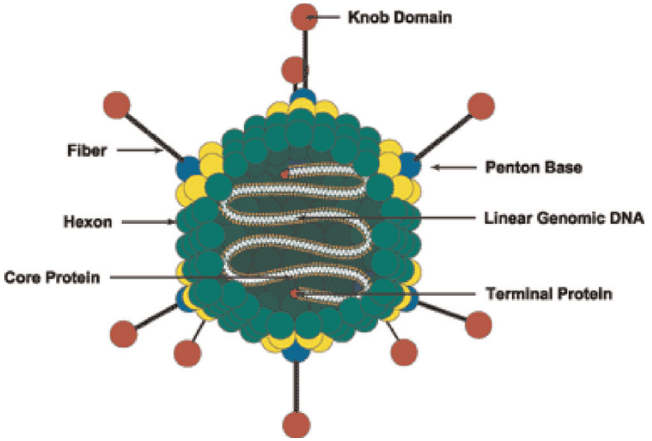
3. Results and discussions

3.1. Plastic antibody synthesis and characterisation

This novel synthesis method was developed by Cranfield University to create high affinity artificial MIP nanoparticle receptors in the presence of target molecules (adenovirus) covalently immobilised on solid microbeads (Altintas et al., 2015b, 2015c). Due to the application of different temperatures, elution of low and high affinity MIP receptors can be separated. High temperatures (60 °C) are sufficient to successfully disrupt the binding interactions between the high affinity receptors with its target template whereas low temperatures (15 °C) are convenient to elute low affinity receptors due to their weak and easily broken interactions with the target. The adenovirus MIPs were synthesised many times and each production batch was characterised using the dynamic light scattering (DLS) method to check the uniformity, quality and size of the MIPs. This technique measures Brownian motion and relates this to particle size. Brownian motion is the random movement of particles due to the bombardment of the solvent molecules which surround them. The larger the particle the slower the movement as it requires more force to move compared to a smaller particle. Temperature is critical and required to be accurately known and controlled during measurement since it influences the viscosity of the liquid which affects Brownian motion. The size of the adenovirus MIP was found as 265 ± 10 nm with the polydispersity index (PDI) of 0.3 and this data was obtained from four separate productions.

Parallel characterisation studies were also conducted for

Table 1
Polymer components used for MIP receptor synthesis.

Component Name	Chemical Structure	Function
N-isopropylacrylamide (NIPAm)		Backbone monomer
N-tert-butylacrylamide (TBAm)		Hydrophobic functional monomer
Acrylic acid (AAc)		Negatively-charged functional monomer
N,*N*′-methylenebisacrylamide (BIS)		Cross-linker
N-(3-aminopropyl)methacrylamide hydrochloride		Secondary monomer to aid MIP formation, allows immobilisation of MIP on SPR chip
Adenovirus		Target template to imprint

vancomycin-MIP and MS2-MIP as control receptors and the size of the particles were found to be 263 ± 10 nm and 217 ± 8 nm with PDI of 0.3, respectively. Polydispersity indexes (PDI) of the measurement were determined which provides an indication about the accuracy of the measurement, the lower PDI the more accurate the measurement. However, this size is only an approximation and may differ from the size particle sizes. Due to this, transmission electron microscopy (TEM) technique was conducted to check the purity and production quality of the MIP receptors in the solution. A clean image was obtained with the expected particle size which is ~265 nm (Fig. 1). After the characterisation and quality determination of the MIP receptors, the amount of the MIPs was also calculated after the freeze-dried particles which appeared in white, slightly opaque transparent compound in tubes. The total yields for each solid-phase production of MIPs was around 22.5 mg that shows the considerable yield amount to be acceptable and indicated effective plastic antibody (MIPs) synthesis.

3.2. SPR detection of adenovirus using plastic antibodies

The high quality MIP receptors were immobilised onto an SPR-based biosensor to investigate the binding affinity, sensitivity and specificity of MIPs against adenovirus. Amine coupling chemistry was applied to attach biomolecules to gold sensor surface strongly. The principle of this method relies on the activation of carboxyl groups on the sensor surface by an EDC/NHS mixture which provides reactive succinimide esters (Altintas et al., 2011, 2012b). First, the SPR sensor chips were coated with mercaptoundeconoic acid (MUDA) for self-assembled monolayer (SAM) formation on the surface. The mixture of EDC/NHS was then used to activate the surface prior to immobilisation of MIP receptors with a concentration of 250 $\mu g\ mL^{-1}$ which was injected on individual sensor spots to the activated surface. This was successfully achieved and optimised.

The adenovirus samples initially prepared in the concentration range of 0.01–20 pM were injected over the immobilised MIPs and a good reproducibility was obtained in a concentration range of 0.02–20 pM with the detection limit of 0.02 pM (8.08×10^6 pfu mL^{-1}) of adenovirus. Three separate spots of Biacore 3000 system were used for the parallel affinity assays and the response achieved from different spots indicates the reliability and sensitivity of the sensor. The concentration dependent sensorgram is shown in Fig. 2a.The results indicate that the sensor reproducibility is reduced at higher concentration of the virus when saturation is reached. Moreover, to confirm the specificity of the binding interaction between adenovirus-MIP and its target (adenovirus), a control virus (MS2 phage) was also prepared in the concentration range of 0.15–20 pM. The non-specific binding of MS2 phage was found in zero level against adenovirus-MIP whereas an excellent response was obtained for target binding (Fig. 2b).

Kinetic data analysis was performed to investigate the dissociation constant (K_D) and determine the binding affinity between adenovirus and its specific MIP based on Langmuir binding model. K_D was calculated as 3.10×10^{-11} M using Biacore 3000 software which indicates high affinity of MIP receptors against its target virus. Therefore, MIP-based adenovirus assay provided a successful output with high sensitivity and specificity and easy-to-use methodology for virus detection without using any signal amplification or complex technique. Overall results of direct adenovirus assay using plastic antibodies were analysed and R^2 value was found as 0.99 (Fig.2c). Moreover, two different MIPs were also imprinted using MS2 phage and vancomycin. Although MS2 virus has a bit similar structure to adenovirus, vancomycin has completely different structure. Despite of structural and natural similarity between MS2 phage and adenovirus, their size and also

coating materials are quite different. MS2 phage is 25 nm whereas adenovirus is 90 nm (Altintas et al., 2015a). In this study, we aimed to compare these two different MIPs (MS2-MIP and vancomycin-MIP) with our target MIP to see the effect of receptors characteristics. Three MIPs were synthesised using completely same protocol expect for templates and immobilised on the sensor surface as described before. The binding affinity of adenovirus against these three MIPs were comparatively investigated by employing SPR biosensor. Vancomycin-MIP having completely different structure from adenovirus-MIP due to the difference between templates (Table 1) showed too low affinity against adenovirus whereas MS2-MIP showed a low level binding. The binding profiles of two control MIPs against adenovirus confirmed the importance of structural, chemical, shape and size properties of templates for imprinting and also the successful output of target-MIP for highly specific and strong interaction with adenovirus as shown in Fig. 2d.

3.3. SPR detection of adenovirus using natural antibodies

Due to their reliability and specificity, natural antibodies provide consistent, well-established recognition and detection of biomolecules. However, the production of these receptors is more complex and includes expensive long-term processes and ethical issues. In this work, adenovirus recognition and detection were alternatively investigated using natural receptors for comparison. The working conditions for adenovirus polyclonal antibody were initially tested. The running buffer, pH of sodium acetate solution to be used for antibody immobilisation, the concentration of antibody and injection time on sensor system were optimised. Based on the preliminary test, a 50 $\mu g\ mL^{-1}$ of adenovirus antibody was prepared in sodium acetate buffer (pH:4.5) to be immobilised on the sensor surface during 3 min injection using phosphate buffered saline-tween (PBST) as running buffer of the microfluidics system. A good level of immobilisation was recorded for the antibody with RU value of 2621 ± 150 and a 3 min injection of surface antibody was sufficient for the signal to reach equilibrium; therefore, the immobilisation time was kept at 3 min for the assay.

The specific recognition and detection of adenovirus were then investigated using direct binding approach in the concentration range of 0.3–20 pM. For cross-reactivity test, MS2-phage was prepared in same conditions as a control analyte to check the specificity of the adenovirus surface antibody against its own target. As it is seen in Fig.4b, an excellent signal was observed for target binding on the surface whereas MS2-phage did not produce any binding response. Moreover, the surface was successfully regenerated using 0.1 M HCl after each adenovirus sample testing. The level of regeneration was recorded as 100% and it did not cause activity loss or degradation (Fig. 3a). The overall results of direct binding assay for adenovirus recognition were analysed by fitting to polynomial trend and the correlation coefficient was found as 0.99 with acceptable standard deviation (Fig. 3b).

To decrease the detection limit of the binding assay, a sandwich method was developed for adenovirus. The amount of detector antibody was initially tested to determine the best concentration and 5 $\mu g\ mL^{-1}$ was selected based on the preliminary results. For the sandwich assay, the sensor surface was immobilised with surface antibody prior to injection of the target analyte. Adenovirus samples were prepared in a serial concentration range of 0.004–0.5 pM and detector antibody (5 $\mu g\ mL^{-1}$) was injected on to the sensor after each adenovirus sample and the surface was regenerated between cycles as shown in the real-time sensorgram (Fig. 3c). The sandwich method increased the sensitivity of the adenovirus sensor from 0.3 pM to 0.008 pM while the efficiency of the regeneration did not decrease despite of more material binding in each cycle (analyte+detector antibody). Overall output of

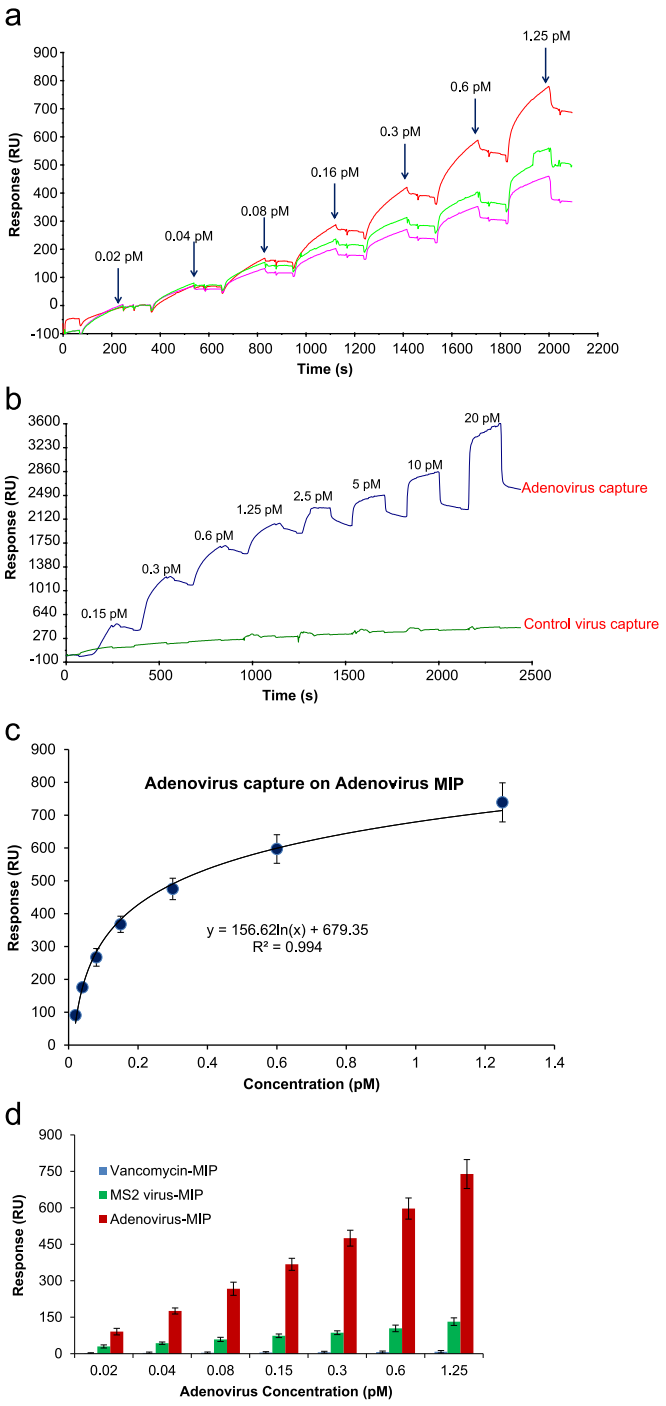


Fig. 2. Direct adenovirus binding assay using plastic antibodies. Real-time sensorgram of adenovirus assay on three simultaneous sensors in the concentration range of 0.02–1.25 pM (a). Cross-reactivity studies using MS2 phage as control virus (b). Overall results of MIP-based direct assay for adenovirus detection in the concentration range of 0.02–1.25 pM (c). Comparison of target MIP with control MIPs. SPR sensors were separately immobilised with three different MIPs (vancomycin-MIP, MS2-MIP and adenovirus-MIP) to perform adenovirus sample testing in the concentration range of 0.02–1.25 pM (d).

sandwich assay was evaluated. The regression analysis was performed in the concentration range of 0.008–0.5 pM as fitting to logarithmic trend and a good reproducibility was achieved with R^2 value of 0.99 (Fig. 3d).

3.4. Comparison of outputs for plastic and natural antibodies

In this paper, a comprehensive research was conducted as

developing three different strategies for adenovirus recognition and detection. MIP receptors were designed and synthesised using a novel solid phase production technique which provides uniform, highly specific and strong recognition elements for the target templates. An excellent output was recorded with these receptors by applying easy-to-use, direct assay approach in a wide concentration range; and adenovirus specific-MIP receptors were successfully developed for the first time. The affinity of these plastics antibodies against adenovirus was high and this was confirmed with kinetic data analysis and a cross-reactivity test. Kinetic data analysis was performed using Biacore 3000 software depending on Langmuir binding model and mathematical values were calculated as dissociation constant (K_D). K_D was determined as 3.10×10^{-11} M which indicates a strong interaction between MIP receptors and adenovirus. Moreover, the limit of detection for MIP-based adenovirus detection was recorded as 0.02 pM and there is no reported work on MIP-based detection of viruses.

MIPs are a popular recognition element for biosensors and can be used for biomolecule detection. The applications of MIPs with the combination of sensor technology show good promise in diagnostics since a limited number of viruses and bacteria have available natural receptors today and the production of these bioreceptors is time consuming entailing expensive procedures and ethical approvals (Hoshino et al., 2010; Jenik et al., 2009). On the other hand, our comparative investigations with natural antibodies for adenovirus detection provide the first research work in the field employing sensor technology. The direct adenovirus assay with antibody was less sensitive compared with MIPs. The detection limit of antibody assays was found to be 15 times higher than the MIP-based direct assay and the kinetic data analysis also confirmed this output with a K_D of 1.41×10^{-9} M. However, the sandwich assay using natural antibody produced the best output for detection limit, kinetic interaction and also regeneration capacity of the surface. K_D was calculated as 2.30×10^{-12} M which represents an excellent affinity with a detection limit of 0.008 pM (Table 2).

When the results are compared with the literature, all three methods developed in this work show superior results with great potential for virus's detection. Meillan et al. (2014) developed a method for Tobacco mosaic virus detection. The virus was immobilised using organic self-assembled monolayer (SAM) followed by analysis with atomic force microscopy (AFM). Due to the larger size of the virus (300 nm), the virus particle can be analysed and visualised using this method. However, the method was unable to make a link between signal and virus concentration. Moreover, the detection principle is not based on affinity and hence the method lacks virus specificity. Another work lists an optical interferometer immunosensor for real-time detection of herpes simplex virus type-1 developed by Ymeti et al. (2007). The virus was investigated in the concentration range of 850–8.5×10^6 pfu $mL^{-1}$and the sensor showed a detection limit of 850 pfu mL^{-1} (virus sized around 150–200 nm). This sensor used a very high concentration of antibody immobilised on the sensor surface and the authors were unable to regenerate the sensor. Our sensor work provided many parameters for the design of a reliable, cost-effective and sensitive biosensor with both natural and plastic antibodies. In another recent work, dengue viruses were detected using fuel cell immunosensors with a Prussian blue nanotube membrane cathode and a platinum mesh anode. The sensor successfully worked in the concentration range of 3–45 pfu mL^{-1}. This fuel cell virus sensor comprised membrane-supported hexacyanoferrate nanotubes with antibody-loaded nanochannels (Wei et al., 2013). Svendsen et al. (2011) used silicon nanowires functionalised with virus protein and applied as a field effect transistor for the detection of Aleutian disease virus. The resistance change measured in real-time and the method could

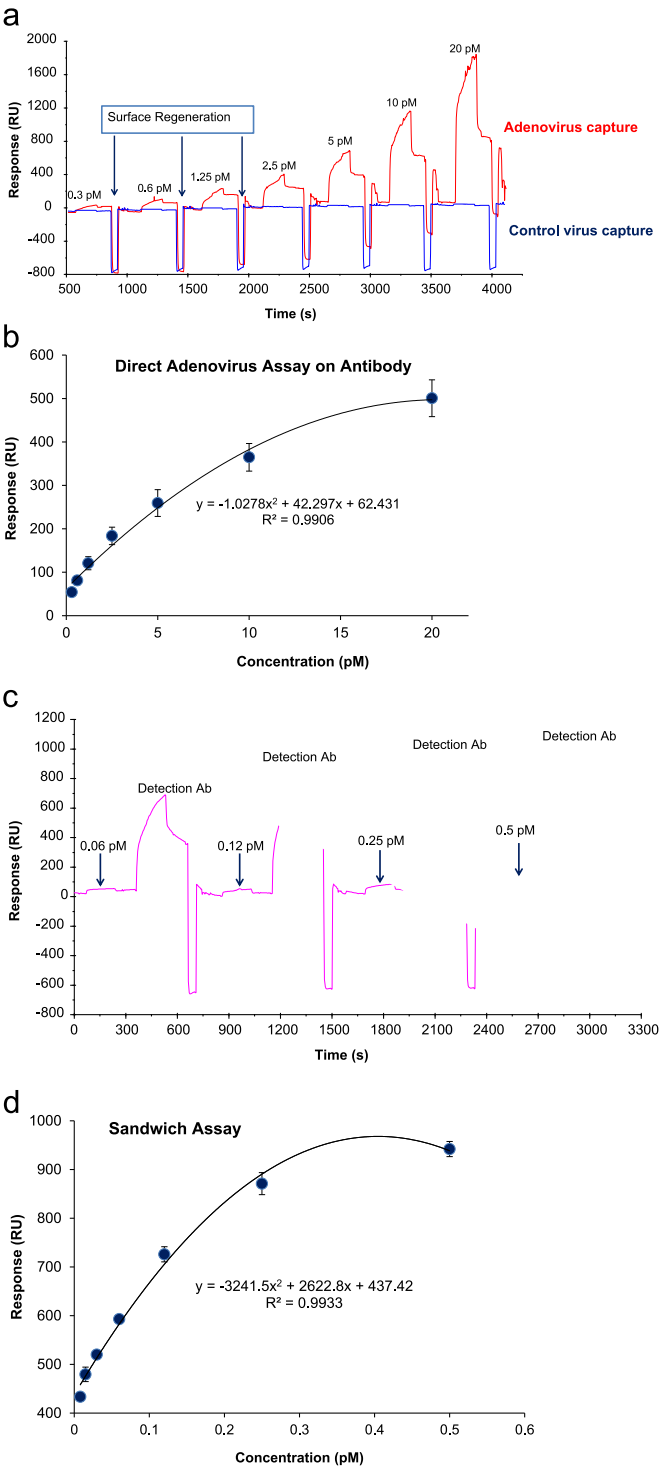


Fig. 3. : Direct and sandwich adenovirus assays using natural antibodies. Real-time sensorgram of direct adenovirus recognition compared to the control virus MS2 (a). Overall results of direct assay using natural antibody in the concentration range of 0.3–20 pM with R^2 value of 0.99 (b). Real-time sensorgram (c) and overall results of sandwich assay (d) in the concentration range of 0.008–0.5 pM.

discriminate the control samples from virus samples based on the recognition by antibody. Human cytomegalovirus and adenovirus were recently studied using ellipsometry and AFM. In this work, antibodies were immobilised on a polymeric surface with gold film coating and the liquid crystals on the surface changed orientation when the target was bound. The viruses were investigated in the concentration range of 1.1×10^5–1.1×10^7 pfu mL^{-1} with a detection limit of 1.1×10^6 pfu mL^{-1} for both

Table 2
Comparison of three different techniques developed in this work.

Assay type	Surface ligand	Investigation range (pM)	Detection limit (pM)	Surface regeneration	Dissociation constant, K_D (M)
Direct	Antibody	0.15–20	0.3	Yes	1.41×10^{-9}
Sandwich	Antibody	0.004–0.5	0.008	Yes	2.30×10^{-12}
Direct	MIP	0.01–20	0.02	N/A	3.10×10^{-11}

viruses (Han et al., 2014). Other biosensors have been developed for other viruses based on antibody or DNA receptors including dengue virus NS1 protein (Dias et al., 2013), influenza virus type A DNA (Tam et al., 2009), feline calcivirus (Connelly et al., 2012), HIV-1 virus (Lee et al., 2013) and bacteriophage MS2 (Dantham et al., 2012). In our work, we achieved a detection limit of 8.08×10^6 pfu mL^{-1} with a real-time, cost-effective, reliable and reusable approach. Moreover, we highlighted the great potential of synthetic MIP receptors which were produced based on a novel solid phase technique as a strong alternative to natural antibodies with a high affinity (K_D: 3.10×10^{-11} M).

4. Conclusions

Although a wide range of analyte targets have been investigated to create molecular imprints, small analytes have dominated in the production of MIPs due to the drawbacks in macromolecule imprinting. These challenges include the structure, size and conformational fragility of macromolecules such as proteins, viruses and bacteria. However, important improvements have been recorded against these limitations in recent years. In this work, we successfully produced MIP based receptors for adenovirus with strong recognition capacity and high specificity for the first time. Natural antibodies were also investigated to compare the important parameters of biosensors-based recognition and detection of viruses. All techniques developed in this study show a great potential for real-time, easy-to-use, cost-effective and highly sensitive virus detection which is so critical not only for environmental testing but also for a lot of important diseases caused by viruses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.07.076.

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