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ORIGINAL PAPER

DNA aptamers against the MUC1 tumour marker: design of aptamer–antibody sandwich ELISA for the early diagnosis of epithelial tumours

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Abstract Aptamers are functional molecules able to bind tightly and selectively to disease markers, offering great potential for applications in disease diagnosis and therapy. MUC1 is a well-known tumour marker present in epithelial malignancies and is used in immunotherapeutic and diagnostic approaches. We report the selection of DNA aptamers that bind with high affinity and selectivity an MUC1 recombinant protein containing five repeats of the variable tandem repeat region. Aptamers were selected using the SELEX methodology from an initial library containing a 25-base-long variable region for their ability to bind to the unglycosylated form of the MUC1 protein. After ten rounds of in vitro selection and amplification, more than 90% of the pool of sequences consisted of target-binding molecules, which were cloned, sequenced and found to share no sequence consensus. The binding properties of these aptamers were quantified using ELISA and surface plasmon resonance. The lead aptamer sequence was subsequently used in the design of an aptamer–antibody hybrid sandwich ELISA for the identification and quantification of MUC1 in buffered solutions. Following optimisation of the operating conditions, the resulting enzyme immunoassay displayed an EC_{50} value of 25 μg/ml, a detection limit of 1 μg/ml and a linear range between 8 and 100 μg/ml for the MUC1 five tandem repeat analyte. In addition, recovery studies performed in buffer conditions resulted in averaged recoveries between 98.2 and 101.7% for all spiked samples, demonstrating the usability of the aptamer as a receptor in microtitre-based assays. Our results aim towards the formation of new diagnostic assays against this tumour marker for the early diagnosis of primary or metastatic disease in breast, bladder and other epithelial tumours.

Keywords MUC1 · Aptamers · ELISA · Diagnostic assay · Immunoassay

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Introduction

MUC1 mucin is overexpressed and aberrantly glycosylated on the surface of cancer cells [1], and has been used successfully as a tumour marker in bladder cancer. It is highly expressed by the majority of human adenocarcinomas and has been associated with poor prognosis [2]. MUC1 is a large cell surface mucin glycoprotein expressed

in the extracellular matrix by most glandular and ductal epithelial cells and some haematopoietic cell lineages [3, 4]. MUC1 is found shed in the bloodstream of adenocarcinoma patients and is the antigen in commercial serum tumour marker assays (CA15-3, Truquant-Br, CASA, CA549, MCA) [5–7].

Aptamer technology [8–10] has emerged in the past decade as a novel approach to evolve highly specialised molecules that can recognise, bind and inhibit a specified target. Aptamers are RNA or DNA ligands, selected for high affinity and specificity against their cognate targets using the SELEX methodology [10]. They have been developed to recognise proteins and receptors [11–14], as well as small molecules [15–19]. Furthermore, aptamers have been used for pure recognition, inhibition, diagnostic and therapeutic applications [20–23] and their high affinity and specificity, plasticity, adaptability and ease of modification give them an advantage over other current technologies [24].

Cancer-related targets, both intracellular (ERK-2, Ras, Raf-1) [25–27] and extracellular [prostate-specific membrane antigen (PSMA), tenascin-C, platelet-derived growth factor and tyrosine kinase] [28–32], have been targeted with aptamers, exhibiting picomolar binding affinities and potential in cancer diagnosis and therapy. The inhibition of the vascular endothelial growth factor receptor by an aptamer mimic of its natural antigen has been achieved and is now in phase I clinical trials for cancer in the USA. This aptamer has been proven to inhibit this receptor both in vitro and in vivo [33–35] and has also been successfully produced as a local therapeutic for the treatment of macular degeneration, constituting the first aptamer therapeutic to receive FDA approval.

The MUC1 recombinant protein used as our target contained five tandem repeats (MUC1-5TR) of the variable tandem repeat (VTR) of the MUC1 protein with a 6-His-tag at the N-terminus of the protein. Our SELEX methodology design is based on affinity chromatography related properties of aptamers [36]. The affinity purification matrix was prepared as previously described [37, 38] and was used for the selection of MUC1-binding aptamer from our combinatorial library. A SELEX library composed of a 25 random- nucleotide region, flanked by two primers to enable amplification, was synthesised to provide 4^{25} different species of DNA aptamers. This size library was selected because it has been reported to be the theoretical minimum to obtain full library diversity and allow formation of all secondary structures known for single-stranded DNA (ssDNA) oligonucleotides. Selected aptamers were evaluated for their binding affinity to MUC1 using a range of techniques, including affinity chromatography, ELISA and surface plasmon resonance (SPR), in a processes similar to that described in our previous work on MUC1 peptides [39]. Oligonucleotide aptamers have been previously selected against synthetic peptides containing the MUC1 tandem repeat sequence and these have been shown to successfully recognise and bind to the native MUC1 at the surface of the MCF7 breast cancer cell lines [39]. Human MUC1 presentation on the surface of the cell is composed of several (more than 100) tandem repeats. The shorter version MUC1-5TRwas engineered to replicate the human MUC1 glycoprotein with a repeated 20 amino acid VTR region, originally to allow for the detailed dissection of rppGalNAc-T1 glycosylation [40]. In our study, the peptide with the five tandem repeats of the MUC1 VTR region presents the antibody-binding epitope more than once, thus allowing the two-site immunoassay design configuration described here. Thus, the potential application of such aptamers in diagnostic assays was evaluated in the design of an aptamer–antibody hybrid sandwich ELISA for the detection of MUC1 in buffered solutions.

Materials and methods

Expression and purification of MUC1-5TR

The MUC1-5TR protein, containing five repeats of the 20 amino acid long VTR sequence of the MUC1 protein and a 6-His-tag at the amino terminus [MUC1-5TR: MGSSHH HHHHSSGLVPR*GSHM(PAPGSTAPPAHGVTSAPDTR) (PAPGSTAPPAHGVTSAPDTR)(PAPGSTAPPA HGVTSAPDTR)(PAPGSTAPPAHGVTSAPDTR)(PAP GSTAPPAHGVTSAPDTR)(PAPG), molecular weight 11,980] was produced and purified according to the method of Brokx et al. [40]. In brief, glycerol stocks of BL21 *Escherichia coli* bacterial cells (Novagen, USA) were streaked fresh on a carbenicillin plate, and a single colony was picked and used to inoculate a 40-ml starter culture. The starter culture (15 ml) was grown overnight at 37 °C until it was turbid. The next day, a 5-ml inoculum from the starter culture was transferred onto 1.5-l preheated Luria–Bertani medium–carbenicillin and incubated at 37 °C. The culture was induced when the optical density at 600 nm reached 0.4, about 2.5 h after inoculation, with 0.4 mM isopropyl β-D-thiogalactopyranoside, the temperature was lowered to 25 °C and the culture was left to grow overnight. Bacterial pellets were recovered by centrifugation at 15,000 rpm for 30 min and MUC1 was purified under denaturing conditions.

Cells were lysed with MUC1 lysis buffer composed of phosphate-buffered saline (PBS) with 8 M urea, 0.35 M NaCl and 10 mM imidazole, 5 ml/g cell pellet. Cell lysis was allowed to proceed with stirring for 2 h at room temperature until the cell pellet was fully dissolved and the

MUC1 protein was purified by nickel nitrilotriacetic acid agarose (Quiagen, Mississauga, ON, Canada). The protein was extensively dialysed, purified by high-performance liquid chromatography and freeze-dried. The sample was further analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with universal dye SimplyBlue (Invitrogen, USA) for the correct band size. The lyophilised powder was calculated to have a molecular mass of 11,800 kDa, which was confirmed by matrix-assisted laser desorption/ionisation mass spectrometry.

DNA library design

A degenerate DNA oligonucleotide library containing a 25-base random sequence flanked by primers on either end, as previously described [41], was used as the starting point for the aptamer selections. The sequence of the library was 5′ GGGAGACAAGAATAAACGCTCAA-(25 N)-TTCG ACAGGAGGCTCACAACAGGC. The forward primer was 5′GGGAGACAAGAATAAACGCTCAA3′ and the reverse primer was 5′AAGCTGTCCTCCGAGTGTTGTCC G3′. To enrich all DNA aptamer species in the original library, a ssDNA pool was generated from the original synthetic pool by large-scale unidirectional PCR. A 400-μl PCR was prepared in a thin-walled PCR tube: aptamer library 4 nmol (100 μl), forward primer 100 nmol (100 μl), reverse primer 4 nmol (100 μl) (supplied by Nottingham University, UK), 10× PCR buffer with 40 μl $MgCl_2$ (MBI Fermentas), deoxyribonucleoside triphosphates at 25 mM of each nucleotide (40 μl) (MBI Fermentas), 15 mM $MgCl_2$ (18 μl) (MBI Fermentas), 8 U Taq DNA polymerase (native form) (4 U initially plus 4 U on the 45th cycle to ensure enzymatic activity) (Sigma Aldrich, UK). Ninety-nine rounds of amplification were performed at 94 °C denaturing (1.5 min), 56 °C annealing (0.5 min) and 72 °C extension (1.5 min) in a thermocycler; a final extension step was included at 72 °C for 10 min and soaking at 4 °C.

Affinity chromatography in vitro selection method

An affinity chromatography based method was used for the in vitro selection of aptamers against the MUC1-5TR recombinant protein.

The method was based on the immobilisation of the MUC1 protein to an NHS-HiTrap Sepharose activated column (1 ml) (Pharmacia, Uppsala, Sweden). The column was functionalised according to the manufacturer's instructions. Protein (3 mg) was immobilised to the column matrix and used for aptamer selection. The amount of protein present after the column functionalisation was determined by UV spectroscopy according to the manufacturer's specifications. Use of the NHS-HiTrap Sepharose activated column would allow the immobilisation of the protein to the column via the amino terminus of the protein.

A stringent system of buffers was designed to produce highly specific ssDNA aptamers. The binding buffer was composed of 100 mM NaCl and 5 mM $MgCl_2$, pH 7.4 and selections were carried out at 37 °C to obtain aptamers for use under physiological conditions. The wash buffer and the elution buffer had similar compositions, but with different concentrations of NaCl (0.15 and 1.5 M, respectively). A wash step with 5 column vol of binding buffer to allow for the washing out of weak binders due to increased stress on the interaction caused by the high volume wash preceded an elution step of 1.5 column vol. The library was panned against the immobilised target MUC1-5TR peptide for ten consecutive rounds at room temperature. In-between each round the library was desalted with a PD-10 desalting column to remove excess salt in the sample, freeze-dried to reduce the sample volume and further amplified to enhance competition by the bound aptamer moieties. After ten rounds of selection, the final pools were cloned into a PCR 21-TOPO vector, and clones were further analysed and sequenced to yield the winning DNA base composition of the final pool of aptamers. Sequencing was provided by Macrogen (Seoul, Korea). Sequences were aligned and the family was analysed by a BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, USA).

Displacement ELISA

ELISA was carried out for assessment of binding and epitope characterisation. In brief, an ELISA microtitre plate was coated with 5 μg MUC1-5TR protein in 100 μl carbonate buffer (0.05 mol/l, pH 9.6) and was left overnight at 4 °C. The wells were subsequently blocked with 100 μl PBS containing 1% (w/v) bovine serum albumin (BSA) for 1 h at 37 °C. The wells were then washed three times with 200 μl PBS containing 0.05% (v/v) Tween-20. A 50-μl aliquot of excess C595 anti-MUC1 monoclonal antibody at a fixed concentration (30 μM in PBS containing 0.05% Tween-20 and 1% BSA) was added per well to ensure saturation of all binding sites and the mixture was incubated for 1 h at 37 °C. The plate was washed and 50 μl of a concentration series of aptamer ranging from 100 to 700 nM in buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 100 mM KCl and 5% glycerol was added to each well and the mixture was incubated for 1 h at 37 °C. The plate was again washed and 50 μl of an anti-mouse secondary antibody peroxidase conjugate (Sigma) was added to the plate and incubated for 1 h at 37 °C. The wells were washed a further three times and colour was developed by addition

of 2,2′-azino-di-(3-ethylbenzothiozolin sulfonic acid) reagent (Sigma).

MUC1 protein biotinylation

The MUC1-5TR recombinant protein was biotinylated using the FluoReporter® biotin-XX protein labelling kit from molecular probes (USA). In brief, 5 mg protein was dissolved in 1 ml carbonate buffer (0.1 M sodium bicarbonate, pH 8.3) immediately before conjugation. Just before use, 2 mg biotin-XX succinimidyl ester was dissolved in 0.1 ml dimethyl sulfoxide at room temperature, vortexed until it had dissolved, added to the protein solution as per the manufacturer's specifications and incubated at room temperature for 2 h. The solution was desalted in a PD-10 column (Pharmacia, Sweden) and tested for biotin levels according to the manufacturer's positive indication kit procedure using the inherent properties of the dye 4′-hydroxyazobenzene-2-carboxylic acid. The levels were measured using UV absorption at 500 nm, according to the manufacturer's instructions, and the difference in absorbance for the biotinylated protein sample in relation to the control (PBS) was calculated.

Surface plasmon resonance

All measurements were performed using a BIAcore 2000 automated machine (BIAcore, Uppsala, Sweden). The principle is based on the optical phenomenon of SPR. The system was operated at 25 °C in running buffer consisting of 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 7.4 or 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4, depending on the selection conditions (see also "Results" and Table 1).

A Sstreptavidin-coated sensor chip (BIAcore SA) was conditioned using 1 M NaCl and 50 mM NaOH for 1 min three times, according to the manufacturer's instructions. Biotinylated protein in running buffer was allowed to interact with the sensor's surface for 3 min. The immobilisation was confirmed by the increase in response units. The ligands (aptamers) were dissolved in running buffer and were injected at a medium pace over the surface at 25 μl/min.

Table 1 Aptamer sequences from the selection against the MUC1 five tandem repeat (MUC1-5TR) recombinant protein

Aptamer	Sequence
MUC1-5TR-1	GAAGTGAAAATGACAGAACACAACA
MUC1-5TR-2	GGCTATAGCACATGGGTAAAACGAC
MUC1-5TR-3	CAAACAATCAAACAGCAGTGGGGTG
MUC1-5TR-4	TACTGCATGCACACCACTTCAACTA

Aptamer families are shown without the flanking primers used for selection
In a 100 mM NaCl 5 mM $MgCl_2$ buffer; elution with 1.5 M salt

After the injection was complete, the complex was washed for an additional 30 s with 1× binding buffer. The chip surfaces were regenerated down to protein level by application of 3 M NaSCN or 1.5 M NaCl, 5 mM $MgCl_2$, pH 7.4 in 30-s pulses. The association and dissociation rate constants of the aptamer–MUC1 complexes were determined using a nonlinear regression analysis of the initial parts of the association and dissociation phases of the sensogram, using Origin software (MicroCal) [42].

Diagnostic sandwich ELISA

Two-site enzyme immunoassays were performed, where the MUC1-5TR analyte was 'sandwiched' between the immobilised 5TR.1 aptamer, biotinylated at the 5′-end (5′biotin-GAAGTGAAAATGACAGAACACAACA, molecular weight 8,169.5, from Sigma-Genosys, Haverhill, UK), and the murine anti-MUC1 C595 antibody (supplied by the University of Nottingham). The bound antibody was subsequently detected using a rabbit anti-mouse immunoglobulin G (IgG) alkaline phosphatase (AP) conjugate (Sigma, Ireland).

Various ELISAs were performed for optimisation of the operating conditions on polystyrene, 96-well, flat-bottom Nunc MaxiSorp Immuno™ plates (Bio-Sciences, Ireland) in the following manner. First, 50 μl per well of the coating reagent—avidin, extravidin or streptavidin (Sigma, Ireland)—was immobilised to the surface of the wells using a 10 mM PBS buffer at pH 7.4. Vacant spaces were then blocked with 200 μl per well of blocking buffer [appropriate blocking reagent in TBS buffer: 8.4 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride HCl, 1.6 mM Trizma base, 137 mM NaCl, pH 7.4). Afterwards, various concentrations of the biotinylated 5TR.1 aptamer and the MUC1-5TR protein (50 μl per well) in 10 mM Tris-HCl, 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$ buffer at pH 7.2 were added successively to the wells. The anti-MUC1 antibody was added to the 8.4 mM Tris-HCl, 1.6 mM Trizma base, 100 mM NaCl buffer and the goat anti-mouse IgG-AP label in 42 mM Tris-HCl, 8 mM Trizma base, 1 mM $MgCl_2$ buffer, both at pH 7.4 (50 μl per well). The substrate consisted of 100 μl per well of 1 mg/ml *p*-nitrophenyl phosphate substrate (Sigma, Ireland) in 100 mM diethanolamine, 50 mM KCl, 1 mM $MgCl_2$ buffer, at pH 9.8. The colour was developed at 37 °C and the absorbance was measured at 405 nm using an ELISA spectrophotometer (microplate autoreader, model EL311, from Bio-Tek Instruments, VT, USA). After each step the plate was incubated for 1 h at 37 °C, and in-between steps the wells were washed three times with 300 μl of washing buffer (42 mM Tris-HCl, 8 mM Trizma base, 100 mM NaCl, and 0.05% v/v Tween-20 at pH 7.4).

For the recovery experiments, buffer solutions (10 mM Tris, 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, pH 7.2) spiked with various analyte concentrations were assayed in duplicate

(n=2) and the observed levels, returned from the optimised competition curves, were compared with the original spiked values to determine the recovery (as a percentage).

Results

Targeting the tumour marker MUC1 antigenic peptides with ssDNA aptamers

Selection of ssDNA aptamers with the ability to recognise a five tandem repeat construct of the MUC1 protein was performed using affinity chromatography matrices from a pool of 4^{25} (approximately 10^{15}) different molecules each consisting of a 25 random-nucleotide region flanked by two fixed primer regions on either side [40]. The MUC1-5TR protein was expressed in *E. coli*, was purified on the basis of previously published protocols [39] and was used directly as a target in aptamer selection processes.

For the selection of aptamers against the MUC1-5TR recombinant protein that recognises the VTR sequence of MUC1, the MUC1-5TR protein was immobilised on functionalised Sepharose, in an affinity chromatography column. The synthetic library was amplified to give a starting pool of ssDNAs where each species was represented by a number of molecules and to ensure no potential high-binding species was lost at the early stages of selection owing to experimental procedures. The pool was incubated with the protein in the affinity chromatography matrix for 1 h at room temperature. Nonbinding or non-specific-binding species were washed from the column during the wash step and binding aptamers were eluted with high salt concentration (1.5 M NaCl). The constant primer regions flanking the random region allowed the amplification of binding species by PCR, and these were subsequently returned to the matrix for the next partitioning step. At each successive round the highest binding species displaced through competitive binding the weaker MUC1 ligands from the column, resulting in a limited pool of high-affinity molecules. Ten consecutive rounds of selection were carried out and after the sixth round the DNA content was monitored on a 3% agarose gel. The aptamers were subsequently cloned and 100 or more clones were analysed and sequenced. The sequences were sorted and aligned using the BioEdit sequence alignment editor (Isis Pharmaceuticals) and were compared with each other to identify conserved regions. Four different aptamer families (Table 1) with conserved regions were identified and purchased for further study.

The four aptamers selected (Table 1) were synthesised chemically and synthetic aptamers were first screened through the selection column to remove any nonbinding sequences generated owing to artefacts in the cloning and sequencing process. The aptamers selected were found to be eluted from the affinity chromatography matrix at 1.3 M NaCl (±0.05 M; Fig. 1).In similar studies, the C595 monoclonal antibody was shown to be eluted from the chromatography matrix at approximately 0.8 M NaSCN [37, 38]. Thus, all four sequences selected were shown to be true aptamers and not amplification errors, which are random and thus appear as single sequences and are not prevalent in large families within the cloned and synthesised aptamers.

Following the initial assessment of binding of aptamers to the MUC1, we utilised the MUC1-5TR protein as a target for the development of diagnostic assays, given that

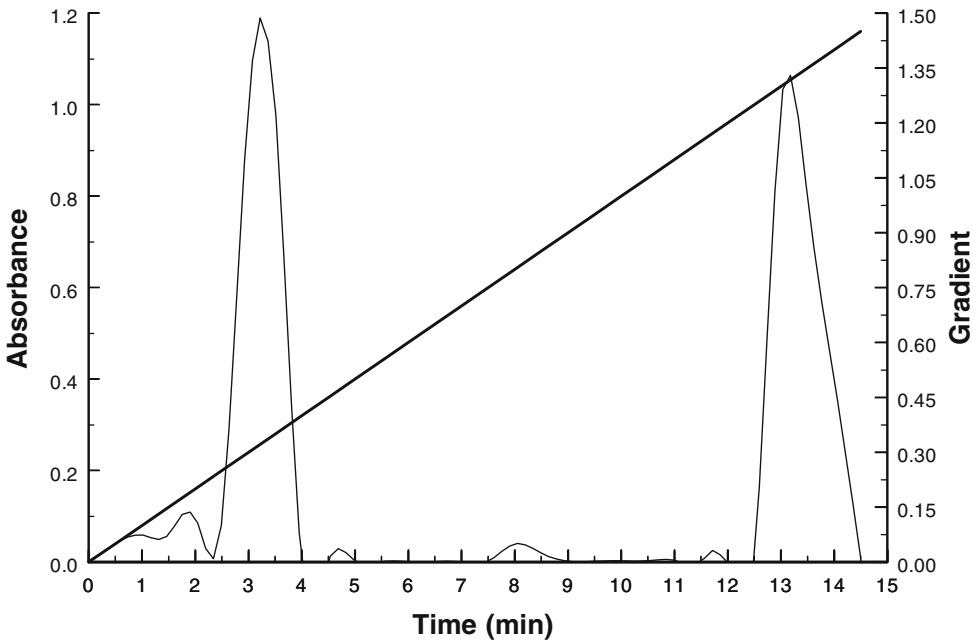

Fig. 1 Elution profile of the 5TR-1 aptamer from the MUC1 five tandem repeat (MUC1-5TR) affinity column using a differential salt gradient (NaCl). Excess aptamer was eluted at the wash step, whereas bound aptamer was eluted from the column at 1.3–1.4 M NaCl

current antibodies to MUC1 are based on the recognition of the nonglycosylated VTR of MUC1. Thus, the aptamers selected for the MUC1-5TR protein were characterised by displacement ELISA and SPR prior to utilising them in the development of a diagnostic assay.

Displacement ELISA assay

To verify the efficacy of the aptamer in binding to the MUC1-5TR protein and competing with the established anti-MUC1 antibody for epitope binding, we used a previously described displacement ELISA [39, 43]. Aptamers against the C595 antibody have been shown to successfully compete for the antibody CDR with the MUC1 peptides. However, it is possible that small unstructured peptides, such as those described in the antibody study, competed more easily than the longer protein construct.

To ascertain the ability of the aptamers to bind to the MUC1-5TR protein on the VTR epitope and displace the C595 antibody, the protein was adsorbed onto the ELISA plate overnight. The C595 antibody was added to the ELISA in excess, to allow saturation and binding to all available protein. Excess antibody was washed away and the relevant aptamers were added to the wells at concentrations ranging between 0 and 600 nM. Colour was developed using a secondary, anti-mouse horseradish peroxidase conjugated antibody. As the protein was immobilised in the wells of the ELISA plate, any antibody that remained bound to MUC1-5TR after the addition of aptamer would interact with the horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin, leading to colour development. The antibodies that were displaced from the protein, as aptamers bound to it, were removed in the various wash steps. A well where no aptamer was added was used as a positive control. Two negative controls were used. One was with buffer only, where no aptamer or antibody was added. The second was based on the addition of a scrambled aptamer instead of the ones selected. The aptamers selected and tested in this ELISA have variable base composition. A scrambled aptamer with base composition 7A, 5G, 7C, 6T has been previously shown not to interfere with the interaction between C595 and MUC1 peptide in this ELISA system [43]. The results (Fig. 2) were plotted as percentage absorbance versus aptamer concentration.

The abilities of the aptamers to displace the C595 antibody from its native antigenic protein appeared very similar between the aptamer species selected and approximately 200 nM of aptamer was enough to achieve 50% or more displacement of the antibody, resulting in the absorbance returning to background levels and further demonstrating the high affinity of the aptamers for the MUC1-5TR protein.

Surface plasmon resonance

To quantify the interaction of the MUC1-5TR recombinant protein with the various aptamers selected and to express it in the form of association and dissociation constants, we utilised SPR with a BIAcore instrument. By plotting the changes of association (units of resonance) and time (seconds), we obtained experimental binding curves and calculated association and dissociation constants.

In our experiments, the biotinylated MUC1 protein was produced (see "Materials and methods") and immobilised on a BIAcore SA sensor chip (gold–streptavidin) and the system was optimised to reduce mass transport, steric hindrance, crowding, avidity and aggregation. This was achieved during the loading of the BIAcore SA chip, by

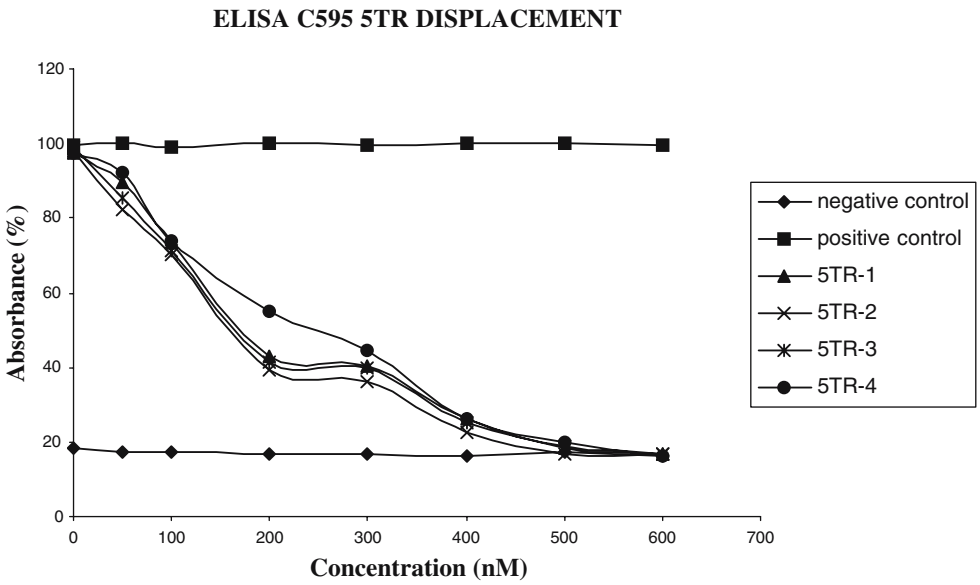

Fig. 2 Displacement ELISA of the C595 anti-MUC1 antibody from the MUC1-5TR protein using the four aptamer species selected. A scrambled aptamer has been previously shown not to interfere with this interaction [43]

adding the protein in low concentrations to allow controlled loading following BIAcore guidelines and calculations for optimal chip load with reference to biomolecule characteristics. A range of protein concentrations, ranging from 1 μM to 1 nM, were tested under different injection times and the optimum conditions used were 100 μl of 1 nM protein in running buffer perfused over each individual channel at a rate of 25 μl/min for 60-s pulse. Chip regeneration to protein level was followed by the subsequent application of a 30-s pulse with a solution of 10 mM NaOH. The aptamers were prepared in binding buffer at 1 μM and were applied with 30-s injections with a 5-s wash step with washing buffer to allow for removal of nonspecific analyte. The chip surfaces were finally regenerated (elution step) by the application of a 10-μl pulse of 1.5 M NaCl, 5 mM $MgCl_2$, pH 7.4.

The experimental BIAcore data were fitted to two mathematical equations using Origin 6.0 (Fig. 3) for the determination of association (k_a) and dissociation (k_d) constants using nonlinear regression analysis.

The fast initial association phase was analysed by nonlinear regression analysis of a one-phase exponential association model expressed by the following equation as described previously [42]:

$$\text{Response} = \text{plateau} + \text{span} \times \{1 - \exp[-K(t - t_0)]\}.$$

The dissociation phase was also subjected to nonlinear regression analysis of a one-phase exponential association model expressed by the following equation [42]:

$$\text{Response} = \text{bottom} + (\text{plateau} - \text{bottom}) \exp[-k_d(t - t_0)].$$

The concentration of free aptamer was assumed to be constant since an excess amount was applied to the system when compared with the amount of MUC1. Therefore K is the same as k_aC+k_d , where C is the total aptamer concentration in the system and k_a is obtained from the following relationship: $k_a = (K-k_d)/C$. K_d was calculated from the following relationship: $K_d = k_d/k_a$. The fit of the data was represented as $\chi 2$.

The binding constants determined are given in Table 2.

The SPR data confirmed high affinity of the aptamers for MUC1-5TR in solution. K_d values in the range 45–85 nM were obtained, but are not, however, as low as those obtained for aptamers against other proteins and peptides, perhaps owing to the unstructured nature of the MUC1 protein. Even so, the association constants obtained are still higher than those obtained for monoclonal antibodies to this protein, thus indicating that the use of aptamers for the recognition of MUC1 in cancer diagnosis may provide more sensitive assays than the currently available antibody-based ones.

When the aptamers were analysed by SPR, their association rates were very fast. However, their dissociation was somewhat slower and two or more pulses of 30 s of elution solution were necessary to completely dissociate the aptamer from the cognate peptides. This has been reported before [30], where the rate of association and the size of the protein where speculated to be the reason. The high association rates could result in a fast rate of reassociation of the aptamer to the peptide, thus causing this effect.

Two-site immunoassay development

The MUC1-5TR protein has five copies of the MUC1 tandem repeat plus an additional four amino acids from the next tandem repeat. It therefore contains more than one epitope for the C595 antibody (RPAP) or the corresponding aptamer (5TR.1), making it an appropriate target for a two-site immunoassay format where it can be 'sandwiched' between the aptamer and the antibody.

Initial attempts to immobilise the antibody and then detect the bound analyte using the aptamer ligand proved

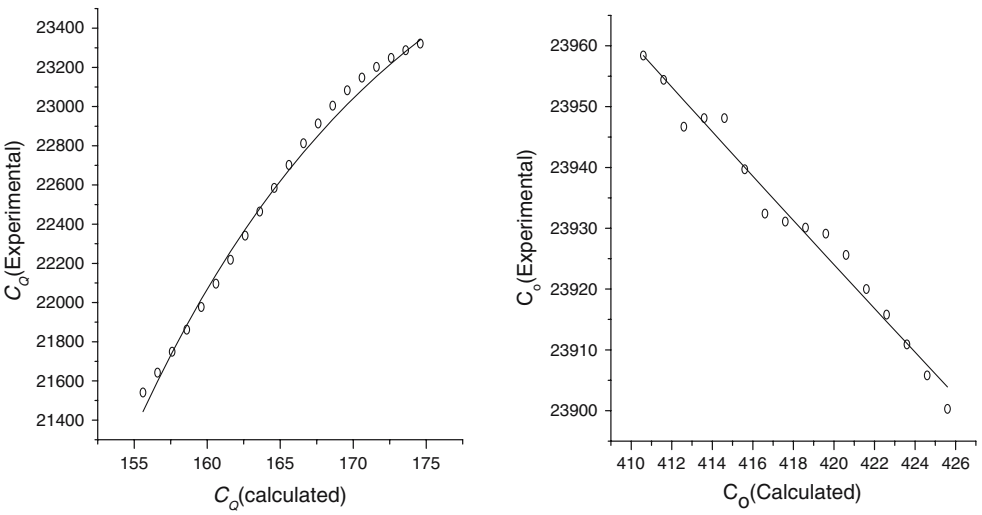

Fig. 3 Fitting of association (*left*) and dissociation (*right*) events measured by surface plasmon resonance for the 5TR-1 aptamer against its target protein MUC1-5TR. Data were analysed using Origin 6.0. C_0(calculated) corresponds to time in seconds. C_0(calculated) corresponds to response units as given by BIAcore 2000 software

Table 2 Association binding parameters resulting from the analysis of the surface plasmon resonance data for the interaction between the selected aptamers and the MUC1-5TR protein

Aptamer	k_a (1/M s)	k_d (1/s)	K_d (nM)	χ^2
MUC1-5TR-1	0.207×10^7	0.00098	47.3	±0.984
MUC1-5TR-2	1.488×10^7	0.01268	85.2	±0.986
MUC1-5TR-3	0.430×10^7	0.00358	83.0	±0.981
MUC1-5TR-4	0.45×10^7	0.00311	69.0	±0.974

ineffective, as the signal generated was below 0.16 AU, even when the antigen and aptamer concentrations used were as high as 10 μg/ml. The success of the mouse C595 antibody immobilisation was confirmed using an anti-mouse IgG–AP conjugate in a simple capture ELISA, where the response obtained reached 1.3 AU for a 1:800 dilution of the coating antibody and 0.85 μg/ml of the enzyme conjugate. The binding efficiency of the C595 antibody towards the MUC1-5TR protein was also established in a different ELISA setup where the analyte was immobilised on the surface of the wells and detected using the C595 antibody and the anti-mouse IgG enzyme conjugate; a signal of about 1.0 AU was acquired for a combination of 1 μg/ml antigen and 1:600 antibody. However, when a similar setup was used with the biotinylated aptamer and an extravidin–AP label in place of the primary and secondary antibody, the response obtained was less than 0.2 AU, even at a 20 times higher antigen concentration. It was thus suggested that the problem with the initial two-site immunoassay format was due to the aptamer being unable to bind to the MUC1 protein, either because of the analyte being structurally restricted as a result of immobilisation (directly or via the antibody) or owing to the close proximity of the biotin tag interfering with the aptamer structure and binding affinity for its MUC1 target.

It was thus decided to reverse the format, where the aptamer would be attached to an extravidin-coated surface and the bound MUC1-5TR would be detected using the C595 antibody and an appropriate anti-species enzyme conjugate, as shown in Fig. 4. In this way we could ensure a more orientated immobilised receptor, avoid any possible structural interference from the biotin moiety and allow the antigen to be less constrained structurally by keeping it in solution.

Initially, the aptamer demonstrated good affinity for MUC1-5TR, providing a sufficient signal of about 1.0 AU after 30 min of colour development, when used at 2 μg/ml and combined with an equal amount of analyte, as shown in Fig. 5. In this case, 20 μg/ml extravidin was used to coat the surface and the bound antigen was detected using a 1:800 dilution of the mouse C595 antibody and 0.85 μg/ml of anti-mouse IgG–AP. A dose-specific response for MUC1-5TR can be observed; however, the changes in signal intensity vary only slightly in relation to the aptamer concentrations investigated. High background values (0.45 AU or less) corresponding to no aptamer or MUC1-5TR added to the wells led us to investigate all possible negative controls to identify any potential nonspecific interactions between the various system components. It was observed that the analyte bound nonspecifically to the coating and blocking reagent, probably through hydrogen-bonding interactions involving its various serine and threonine residues (data not shown). It was thus decided to optimise the system for the various reagents used, in an attempt to minimise any undesired reactions leading to elevated controls.

Three different coating reagents were tested with the biotinylated aptamer for their binding efficiency and nonspecific binding towards the MUC1-5TR antigen, C595 antibody and enzyme label: avidin, extravidin and

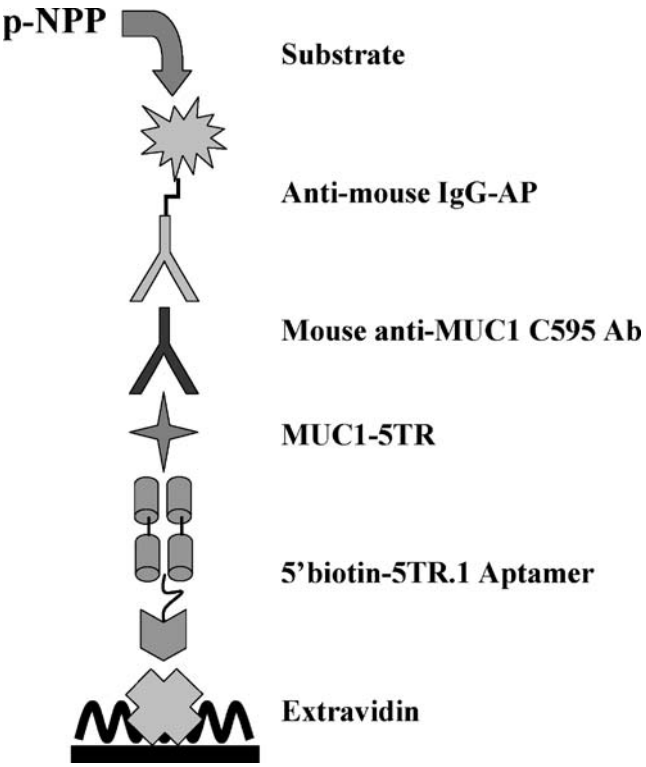

Fig. 4 A two-site immunoassay format for MUC1-5TR, with the biotynilated aptamer immobilised via extravidin and the antibody captured by the analyte and subsequently detected by anti-species enzyme conjugate. *Ab* antibody, *AP* alkaline phosphatase, *IgG* immunoglobulin G, *p-NPP* *p*-nitrophenyl phosphate

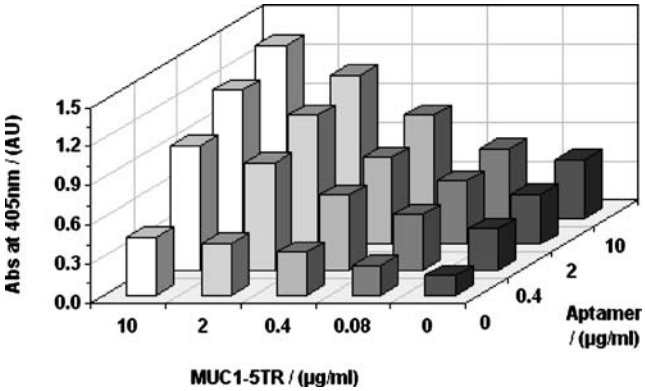

Fig. 5 Results of a two-dimensional immunoassay, where the MUC1-5TR protein and aptamer concentrations are varied simultaneously; the surface was coated with 20 µg/ml extravidin, the bound antigen was detected using a 1:800 dilution of the mouse C595 antibody and 0.85 µg/ml anti-mouse IgG-AP, and the absorbance levels were measured after 30 min of colour development using a p-NPP substrate

streptavidin. It was observed that avidin produced the lowest signals and highest blanks of all the reagents throughout the concentration range investigated, possibly owing to its glycosylated side chains and basic nature (pI 10.5) leading to undesired reactions. Extravidin and streptavidin, both nonglycosylated proteins with near-neutral isoelectric points, performed in a similar manner, with extravidin producing a slightly elevated specific response and signal-to-blank ratio in comparison, even though the nonspecific binding of the antigen to the coating reagent is noticeable in both cases. Furthermore, it was demonstrated that the use of PBS immobilisation buffer at pH 7.4 was associated with higher responses and lower nonspecific interactions between the analyte and extravidin, resulting in a signal-to-blank ratio elevated by up to 66% compared with the results obtained with carbonate–bicarbonate buffer at pH 9.6 (data not shown). Thus, an optimum concentration of 20 µg/ml extravidin in PBS immobilisation buffer, corresponding to the saturation level of the well surface, was chosen for subsequent experiments.

Blocking tests were also carried out, comparing the performance of several reagents, including proteins such as BSA, casein and dry milk protein, small compounds such as L-lysine, and nonionic detergents such as Tween-20, in either PBS or TBS buffer. It was found that 1% (w/v) BSA in 10 mM TBS buffer, pH 7.4 was associated with the lowest nonspecific binding towards the analyte, C595 antibody and enzyme conjugate, resulting in signal-to-blank ratios elevated by 16–83%, and it was subsequently used (data not shown). Finally, investigations were made regarding the optimal aptamer, C595 antibody and enzyme label concentrations and buffer types, as well as the buffer protein content (BSA) so as to minimise nonspecific binding effects even further. The most favourable conditions, namely 10 µg/ml aptamer in 10 mM Tris, 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$ buffer at pH 7.2, a 1:800 dilution of the C595 antibody in 10 mM TBS buffer and 0.85 µg/ml anti-mouse IgG–AP in 50 mM Tris, 1 mM $MgCl_2$ buffer, both at pH 7.4 containing 0.1% (w/v) BSA, were combined to obtain curve A in Fig. 6.

The data were analysed on the basis of the following sigmoidal dose–response equation to obtain best-fit parameters, such as the EC_{50} and Hill slope values:

$$\text{Abs} = \text{minimum} + (\text{maximum} - \text{minimum})/1 + 10^{[(\log \text{EC}_{50} - C) \times \text{Hillslope}]}.$$

It was found that curve A corresponded to an EC_{50} value of 79 µg/ml, a detection limit of 6 µg/ml and a linear range between 26 and 212 µg/ml for the synthetic MUC1-5TR protein. Moreover, this curve was related to a favourable Hill slope of 1.002, good accuracy (R^2=to 0.998), an average relative standard deviation (RSD) for all points of the curve of 5.0% and a background of 0.3 AU when no analyte was present, demonstrating a reduction of 33% in nonspecific binding compared with the results in Fig. 5.

In order to improve the sensitivity of the assay, the concentration of extravidin was gradually decreased from 20 to 4.0 µg/ml, while maintaining a fixed aptamer concentration. The results presented in Table 3 show that a reduction in concentration by 50% led to an improvement in the EC_{50} value by almost 30%. On the other hand, all concentrations below 10 µg/ml generated curves with similar characteristics, possibly because lower concentrations of extravidin allow more contact between the analyte and the blocking reagent, thus intensifying the effect of nonspecific binding between MUC1-5TR and BSA.

The concentration of the immobilised aptamer was also reduced to 8, 5 and 2.5 µg/ml, while keeping a fixed

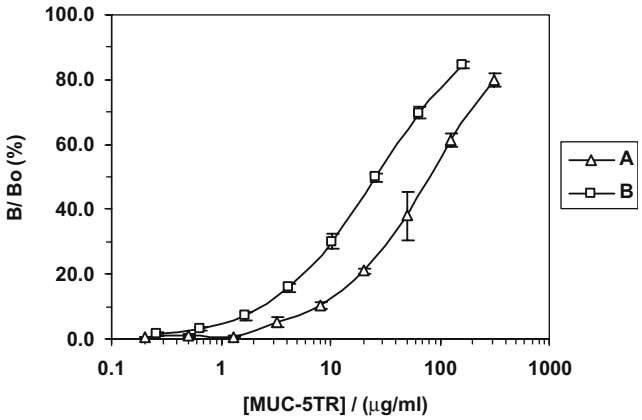

Fig. 6 Effect of a 50% decrease of the immobilised extravidin and aptamer concentration on curve sensitivity; curve *A* corresponds to 20 and 10 µg/ml, while curve *B* corresponds to 10 and 5 µg/ml extravidin and aptamer respectively; B/B_o is the ratio of the average response *B* from two (curve *A*) or eight assays (curve *B*) performed in duplicate (N=2 or 8, n=2), to the maximum response B_o obtained when all the receptor binding sites have been saturated, after having subtracted the assay background corresponding to no analyte being present

Table 3 Comparison of the best-fit parameters for MUC1-5TR two-site immunoassays corresponding to different extravidin or aptamer concentrations

	Extravidin (μg/ml)						Aptamer (μg/ml)			
	20	16	12.5	8.0	6.4	4.0	10	8	5	2.5
Hill slope *k*	1.000	1.020	1.140	1.080	1.020	1.070	0.948	1.020	0.968	0.978
EC_{50} (μg/ml)	79	67	60	56	53	54	56	49	45	48
Range (μg/ml)	26–212	20–200	18–180	18–172	17–172	17–168	22–115	13–145	12–129	12–139
RSD[a] (%)	5.0	3.3	2.5	2.6	1.8	2.2	3.6	5.0	2.3	5.3
R^2	0.9990	0.9990	0.9990	0.9975	0.9985	0.9990	0.9990	0.9980	0.9990	0.9970

Average of two assays performed in duplicate (N=2, n=2). Absorbance measured at 405 nm after 30 min of colour development
[a] Average relative standard deviation (RSD) for all data points of each curve

extravidin concentration, and the results were compared with those of the previous assay corresponding to 10 μg/ml aptamer in Table 3. It was shown that reduction of the aptamer concentration from 10 to 5 μg/ml improved the EC_{50} value by 20%. However, further decrease had no effect on the sensitivity of the assay, possibly owing to the existence of nonspecific binding between the analyte and extravidin, which becomes more pronounced when the aptamer concentration used is not high enough for the aptamer to occupy all available binding sites on the coating reagent.

Therefore, combining the extravidin and aptamer concentrations of 10 and 5 μg/ml respectively, based on the above best-fit analyses results, we performed the assay repeatedly to ensure reproducibility and to standardise the parameters. The curve B in Fig. 6 represents the average response of eight such immunoassays (N=8) performed in duplicate (**n**=2) on separate days. Each point was normalised to 100% in order to compensate for some slight absorbance variations from day to day, mainly due to differences in colour development time. B/B_o is therefore the ratio of average response B from 16 values (N=8, n=2), to the maximum response B_o obtained when all the receptor binding sites have been saturated, after having subtracted the assay background corresponding to no analyte being present. The EC_{50} value associated with this curve is 25 μg/ml, the detection limit is 1 μg/ml, and the linear range is between 8 and 100 μg/ml MUC1-5TR. Additional characteristics include a favourable Hill slope of 0.924, good accuracy (R^2=0.996), minimum error (average RSD for all points of the curve of 3.5%) and a background of 0.25 AU in the absence of antigen, corresponding to a reduction of 44% in nonspecific binding compared with the results in Fig. 5.

Having developed an optimised reproducible immunoassay, we decided to measure spiked samples using various concentrations of MUC1-5TR within the linear range mentioned above (8–100 μg/ml). All samples were assayed in duplicate in buffer conditions (10 mM Tris buffer containing 100 mM NaCl, 100 mM KCl and 5 mM $MgCl_2$ at pH 7.2) and the estimated concentrations returned from the optimised curves were compared with the original spiked values to determine the recovery (as a percentage). The results for 32 samples (four samples and eight assays) are presented in Table 4. The mean sample concentrations, interpolated using the sigmoidal dose–response curve, showed recoveries that varied between 91.4 and 113.2%, with averaged results for each sample between 98.2 and 101.7%, demonstrating the ability of the immunoassay developed to successfully detect concentrations between 8 and 100 μg/ml MUC1-5TR.

Discussion

The MUC1 glycoprotein has been a molecule of interest for the last 20 years and has been found to be a valuable diagnostic tool, with commercial assays being currently available. MUC1 has been implicated in a variety of cancers, including breast, stomach, lung, prostate and colorectal cancers. Furthermore, MUC1 has been identified as an important upregulated tumour marker with good immunogenic properties derived from the incorrect and low glycosylation pattern that is present in neoplastic cells but not in normal cells, allowing the exposure of the protein core. Several biological molecules have used this deregulation in the glycosylation pattern to target the protein core of MUC1 with some success. We have used the same rationale to target the exposed protein core, in the form of a recombinant protein with five tandem repeats of the VTR sequence of MUC1 using DNA aptamers selected in affinity chromatography matrices based on the SELEX methodology.

The selection towards the MUC1-5TR protein yielded aptamer sequences, which were subsequently shown to interact with their chosen target in affinity chromatography assays. Aptamers against the nonglycosylated protein were further characterised by ELISA and SPR and were found to inhibit antibody binding to MUC1 and to present dissoci-

Table 4 Recovery experiments performed in buffer conditions [10 mM tris(hydroxymethyl)aminomethane, 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$ at pH 7.2] using the optimised two-site immunoassay for MUC1-5TR

Real concentration (μg/ml)		100.0	50.0	32.0	20.0	12.5
1	Concentration	102.8	55.0	29.8	20.5	
	Recovery (%)	102.8	109.9	93.1	102.3	
2	Concentration	93.5	51.4	33.3	21.6	
	Recovery (%)	93.5	102.8	104.0	108.1	
3	Concentration		48.8	36.2	19.2	11.4
	Recovery (%)		97.5	113.2	96.2	91.4
4	Concentration		53.8	30.2	19.2	12.3
	Recovery (%)		107.7	94.2	96.2	98.6
5	Concentration		47.0	29.6	20.5	11.6
	Recovery (%)		94.0	92.7	102.6	92.5
6	Concentration		45.9	30.3	20.6	11.7
	Recovery (%)		91.8	94.8	103.0	93.8
7	Concentration		54.2	35.2	21.8	13.6
	Recovery (%)		108.4	110.1	109.1	108.9
8	Concentration		48.0	33.3	19.2	13.3
	Recovery (%)		95.9	104.0	96.2	106.2
Average recovery (%)		98.2	101.0	100.8	101.7	98.6

ation constants in the nanomolar range. This is in good agreement with K_d values obtained for aptamers against other targets. Such examples include the DNA aptamers to thrombin with K_d=25–200 nmol/l [11, 44], RNA aptamer to PSMA with K_d=2 nmol/l [28], RNA aptamer to tenascin-C with K_d=5 nmol/l [29] and RNA aptamer to *Trypanosoma cruzi* cell surface receptor with K_d=172 nmol/l [45]. Again, aptamers appear to have binding properties that compare well with those reported for monoclonal antibodies against MUC1, which have been shown to have K_d values in the millimolar to nanomolar range depending on the antibody (affinities of MUC1 antibodies to MUC1 have been reviewed in [46]). Our aptamers were all found to have K_d values in the nanomolar range, which would allow them to successfully compete with their antibody counterparts. Furthermore, we have previously demonstrated that aptamers selected against synthetic peptides containing the VTR antigenic epitope of the protein (APDTRAPG) can successfully recognise the actual MUC1 protein expressed at the surface of cancer cells (MCF-7 breast cancer cell lines) and potentially used for immunostaining of tumour tissues [39].

Current immunoassays detecting MUC1 are sensitive for predicting relapse in breast cancer, but they are not in widespread use in the follow-up of this disease. It has therefore been proposed [47] that aptamers may offer this additional advantage in improving the sensitivity of such immunoassays. The increased affinity and selectivity obtained by the aptamers selected for MUC1 may offer this increased sensitivity that is necessary.

With this premise, we tested the selected aptamers as potential diagnostic reagents in sandwich ELISAs for the detection of MUC1 in buffered solutions. The current work demonstrates the usability of aptamers as receptors in microtitre-based assays, following careful selection of reagents and optimisation of the conditions to be used throughout the assay, such as reagent concentration, buffer type and protein inclusion in the antibody and enzyme conjugate buffers for reduced nonspecific binding. Under controlled circumstances, the aptamer resulted in a highly accurate and reproducible enzyme immunoassay, displaying a R^2=0.996, a favourable Hill slope of 0.924 and averaged RSD for all points of the curve of 3.5%. In addition, recovery studies performed in buffer conditions resulted in averaged recoveries between 98.2 and 101.7% for all spiked samples. However, the immunoassay developed had a low sensitivity, demonstrating a detection limit of 1 μg/ml, and a linear range between 8 and 100 μg/ml for the MUC1-5TR analyte. The sensitivity of this assay could not be compared with that of the standard CA15-3 assay owing to the fact that CA15-3 is measured in units per millilitre and this can not be related to micrograms per millilitre because it is not possible to weigh or measure MUC1. However, the sensitivity observed in this assay is relatively low compared with the sensitivities observed for other tumour markers (such as prostate-specific antigen), which are often in the range of 0.1–1 μg/l. This disadvantage was not related to the binding efficiency of the aptamer, but was a result of undesired interactions between the analyte and the coating and blocking reagent. Unfortunately, optimisation of the coating and blocking step with different types of reagents and buffers did not reduce the non-specific-binding effect. The only viable option was to maintain the biotinylated aptamer concentration at levels high enough for the aptamer to occupy all

available epitopes on the extravidin molecule and to obstruct the access of MUC1-5TR to the surface components, thus preventing further improvement of the assay sensitivity. The assay could further benefit from testing linkers of different length, using either a series of thymines or a carbon chain linker to increase the distance of the aptamer to the surface, thus perhaps increasing its flexibility and ability to interact with MUC1. Finally, testing the aptamer with either spiked serum samples or samples from cancer patients would require the use of modified aptamers, either containing a fluoro group or a flipped base on the free end of the aptamer, to avoid nuclease degradation.

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