Single-stranded DNA aptamer targeting and neutralization of anti-D alloantibody: a potential therapeutic strategy for haemolytic diseases caused by Rhesus alloantibody

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Background. Rhesus (Rh) D antigen is the most important antigen in the Rh blood group system because of its strong immunogenicity. When RhD-negative individuals are exposed to RhD-positive blood, they may produce anti-D alloantibody, potentially resulting in delayed haemolytic transfusion reactions and Rh haemolytic disease of the foetus and newborn, which are difficult to treat. Inhibition of the binding of anti-D antibody with RhD antigens on the surface of red blood cells may effectively prevent immune haemolytic diseases.

Materials and methods. In this study, single-stranded (ss) DNA aptamers, specifically binding to anti-D antibodies, were selected via systematic evolution of ligands by exponential enrichment (SELEX) technology. After 14 rounds of selection, the purified ssDNA was sequenced using a Personal Genome Machine system. Haemagglutination inhibition assays were performed to screen aptamers for biological activity in terms of blocking antigen-antibody reactions: the affinity and specificity of the aptamers were also determined.

Results. In addition to high specificity, the aptamers which were selected showed high affinity for anti-D antibodies with dissociation constant (K_d) values ranging from 51.46±14.90 to 543.30±92.59 nM. By the combined use of specific ssDNA aptamer 7 and auxiliary ssDNA aptamer 2, anti-D could be effectively neutralised at low concentrations of the aptamers.

Discussion. Our results demonstrate that ssDNA aptamers may be a novel, promising strategy for the treatment of delayed haemolytic transfusion reactions and Rh haemolytic disease of the foetus and newborn.

Keywords: single-stranded DNA, aptamer, RhD alloantibody, delayed haemolytic transfusion reactions, haemolytic disease of the newborn.

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Introduction

Rhesus (Rh) is the second most common blood group system after the ABO blood group[1]. RhD, also known as cluster of differentiation 240D (CD240D) and encoded by the *RHD* gene, is the most important antigen of the Rh blood group system because of its strong immunogenicity. Individuals can be divided into RhD-positive and RhD-negative according to their expression of D antigen[2]. When RhD-negative individuals are exposed to RhD-positive blood, they may produce anti-D antibodies (IgG), resulting in delayed haemolytic transfusion reactions. Anti-D antibodies also play a crucial role in haemolytic disease of the foetus and newborn and used to be a major cause of foetal death. Despite immunosuppressive therapy with anti-D immunoglobulin prophylaxis, D alloimmunisation in pregnancy still occurs and haemolytic disease of the foetus and newborn remains a clinical concern[3]. The treatment of this disease includes intrauterine transfusion for RhD-negative women in pregnancy and exchange transfusion for foetuses[4-6]. The side effects of these treatments include transfusion-transmitted infections, infantile retinopathy and bronchopulmonary dysplasia[7,8].

Aptamers are synthetic oligonucleotide molecules with stable three-dimensional structures capable of combining with specific targets via complementation, ensuring high affinity and specificity[9]. Aptamers are usually screened from random oligonucleotide libraries consisting of numerous oligonucleotides. The process is known as systematic evolution of ligands by exponential enrichment (SELEX)[10,11]. *In vitro* selection of single-stranded (ss) DNA aptamers via SELEX is an iterative process incorporating three basic steps: binding of aptamers with the target molecule, separation of the aptamer/ligand complex from non-specific aptamers, and amplification of specific aptamers by polymerase chain reaction (PCR)[12]. Aptamers with high affinity for target molecules are usually enriched after 4-12 rounds of selection[13].

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SELEX technology is beginning to be used increasingly in clinical research, including treatment and diagnosis[14]. Pegaptanib (Macugen, San Dimas, CA, USA), which binds to vascular endothelial growth factor, was the first aptamer drug approved by the Food and Drug Administration in 2004 for the treatment of age-related macular degeneration[10,15]. Because of the ease of synthesis, high stability, biological compatibility, ease of modification, low immunogenicity and low toxicity of aptamers[16], an increasing number of aptamer drugs have been developed for infectious diseases, e.g. human immunodeficiency virus, and chronic conditions including cancer[17-19]. Aptamers that bind to antibodies of the Rh blood group could be a novel strategy for targeted treatment of delayed haemolytic transfusion reactions. In this study, we identified ssDNA aptamers that act as "blocking ligands" to prevent the combination of anti-D allo-antibodies with RhD antigens expressed on the surface of red blood cells. We suggest that the aptamers identified could have therapeutic potential in the prophylaxis and treatment of both delayed haemolytic transfusion reactions and haemolytic disease of the foetus and newborn.

Materials and methods

Chemicals and materials

The initial ssDNA random library and all primers used are shown in Table I and were synthesised and purified using high performance liquid chromatography by TaKaRa (Dalian, China). Reagents for symmetric PCR amplification (Taq DNA polymerase, dNTP, $MgCl_2$, 10×buffer) and streptavidin-coated magnetic beads were purchased from Promega (Madison, WI, USA). Reagents for asymmetric PCR amplification (AmpliTaq Gold Fast PCR master mix), protein A-coated Dynabeads, microspheres (Aldehyde/Sulfate Latex beads, 4% w/v, 9.0 μm) and Hulamixer were from Life Technologies (Carlsbad, CA, USA).

Blood-typing antibodies including anti-D IgG monoclonal antibody, anti-B IgM antibody and reference panel red blood cells were from Shanghai Hemo-pharmaceutical & Biological Co., Ltd. (Shanghai, China). Anti-Fy^a IgM antibody and anti-human IgG monospecific antiglobulin reagent were from Sanquin (Amsterdam, The Netherlands). Human IgG whole molecule and human IgM (myeloma) whole molecule were from Rockland (Limerick, PA, USA). Protein A was from Sigma (Shanghai, China). Fluoroscein isothiocyanate (FITC)-conjugated anti-protein A antibody, and FITC-conjugated anti-bovine serum albumin (BSA) antibody were from GeneTex (San Antonio, TX, USA). FITC-conjugated goat anti-human IgM antibody and FITC-conjugated goat anti-human IgG antibody ($F[ab']_2$ fragment) were from AbD Serotec (Kidlington, UK).

ssDNA aptamers and ssDNA aptamers labelled with Alexa Fluor 488 were synthesised by Sangon Biotech Co., Ltd. (Shanghai, China). Buffers included HEPES buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.02% Tween 20, pH 7.4), MES (4-morpholine ethane sulfonic acid) buffer (0.025 M, pH 6.0) and phosphate-buffered saline (PBS: 0.1 M, pH 7.2). All reagents for the buffers and BSA were from Amresco (Solon, OH, USA).

Preparation of selection beads and detection microspheres

Magnetic separation was used in the SELEX process. Protein A-coated super paramagnetic Dynabeads can covalently couple with anti-D antibody via the Fc region, and are easily collected by magnetic separation methods. Anti-D-coated magnetic beads offer a simple and effective method to separate ssDNA bound to anti-D from unbound ssDNA, which is a key step in the SELEX elution process[20]. Uncoated magnetic beads were used to remove ssDNA aptamer candidates non-specifically bound to beads. For detection, microspheres were more suitable for flow cytometry to measure the fluorescence intensity of fluorophore-labelled antibodies and proteins.

Anti-D-coated magnetic beads were prepared using protein A-Dynabeads according to the manufacturer's protocol. Uncoated magnetic beads for counter selection were prepared in a similar manner.

Table I - Sequences of ssDNA random library and primers.

Kind	Sequence (5′→3′)	Length (nt)
ssDNA random library	AGAGACGGACACAGGATGAGC-N40-CCTTCCCCAAGACAGCATCCA	82
symmetric PCR forward primer	biotin-AGAGACGGACACAGGATGAGC	21
symmetric PCR reverse primer	biotin-TGGATGCTGTCTTGGGGAAGG	21
asymmetric PCR forward primer	AGAGACGGACACAGGATGAGC	21
asymmetric PCR reverse primer	biotin-TGGATGCTGTCTTGGGGAAGG	21
qPCR forward primer	AGAGACGGACACAGGATGAGC	21
qPCR reverse primer	TGGATGCTGTCTTGGGGAAGG	21

ssDNA: single-stranded DNA; PCR: polymerase chain reaction; qPCR: quantitative real-time PCR.

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Detection microspheres coated with antibody or protein were prepared by mixing 500 μL aldehyde/ sulfate latex beads with 1 mL (~5 mg/mL) of each antibody (anti-A, anti-B, anti-D, anti-Fy^a, human IgG, human IgM) or protein (protein A or BSA) in 2 mL of MES buffer. The mixtures were incubated overnight under rotation at room temperature. Antibody- or protein-coated beads were washed three times in PBS, and re-suspended in 2 mL PBS. Uncoated microspheres for blank controls were prepared in a similar manner.

SELEX procedure

The SELEX procedure was performed as described previously by Jeffrey *et al.*[20] with some modifications to increase the selection stringency (Figure 1). The SELEX procedure is described in Online Supplementary Table SI. In brief, the selected ssDNA library (initial pool size 10^{15}) was denatured at 95 °C for 10 minutes and then cooled on ice for at least 15 minutes. The mixture of ssDNA library and counter selection magnetic beads (in a reaction volume of 2 mL HEPES-T) was incubated at 37 °C for 30 minutes. The supernatant was transferred into a fresh microtube containing selection beads and incubated for the appropriate time as shown in Online Supplementary Table SI. The selection beads combined with ssDNA were washed three times with HEPES, then re-suspended in nuclease-free water to a concentration of 3 μg/μL as templates for PCR, in order to perform quantitative analysis and generate an ssDNA sub-library.

ssDNA sub-library generation and purification

The mixture was purified further in order to remove non-specifically bound ssDNA. Amplification of isolated ssDNA from the initial selection process is needed to generate a sub-library with enough copy numbers of ssDNA. Our laboratory has developed an indirect purification method to enrich specific ssDNA using PCR in sequential rounds including three basic steps: (i) symmetric PCR with biotinylated primers was carried out to provide enough templates for asymmetric PCR; (ii) asymmetric PCR was carried out with an excess of non-biotinylated forward primer and a limited concentration of biotinylated reverse primer in order to generate ssDNA; thus, both double-stranded (ds) DNA and the by-products produced during PCR cycles were biotinylated; and (iii) dsDNA and by-products were eliminated via streptavidin magnetic beads. In each round, this method provided a high yield and high quality ssDNA sub-library for the subsequent rounds[21].

As magnetic beads do not affect PCR amplification efficiency, symmetric PCR was performed on selection beads combined with ssDNA as a template, thus minimising ssDNA losses at each stage of purification[22,23]. PCR was carried out in a 50 μL reaction volume which consisted of 10 μL template, 1×PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0), 1.5 mM of $MgCl_2$, 400 μM of each dNTP, 2.5 U of Taq DNA polymerase, and 50 pmol of each biotinylated primer. The PCR protocol was as follows: initial denaturing at 94 °C for 5 minutes; followed by 11 cycles of 94 °C for 30 seconds, 59 °C for 30 seconds, and 72 °C for 30 seconds; followed by a final 5-minute elongation stage at 72 °C.

Asymmetric PCR was performed in a 20 μL reaction volume which consisted of 1 μL symmetric PCR product as a template, 2 μL forward primer (10 pmol/μL) and 2 μL biotinylated reverse primer (0.5 pmol/μL) (primers at 20:1 ratio), and 10 μL of AmpliTaq Gold Fast PCR master mix. The asymmetric PCR protocol was as follows: initial denaturing at 96 °C for 10 minutes; followed by 30 cycles of 96 °C for 3 seconds, 59 °C for 3 seconds and 68 °C for 3 seconds; and then, a final 10-second elongation at 72 °C.

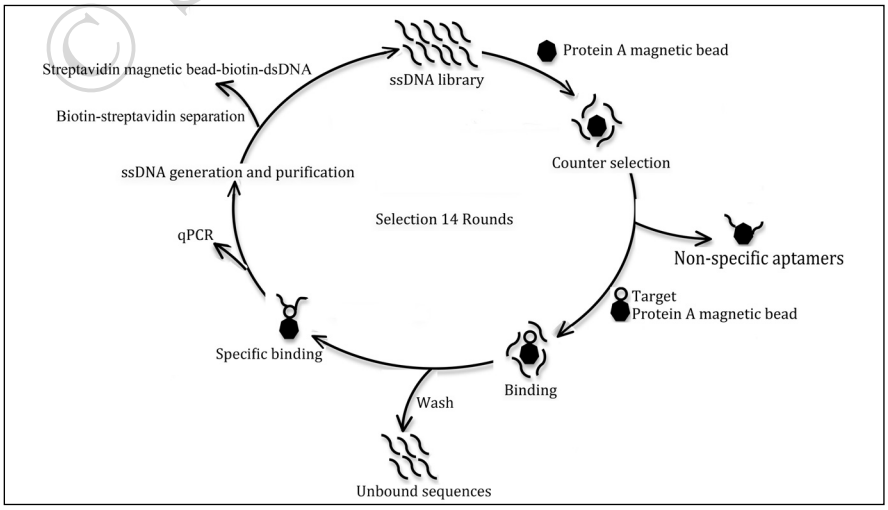


Figure 1 - Schematic representation of the SELEX protocol.
PCR: polymerase chain reaction; ssDNA: single-stranded DNA.

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Streptavidin-coated magnetic beads (10 mg/mL) were added to the asymmetric PCR products, and the mixture was incubated at room temperature for 20 minutes in order to eliminate biotinylated dsDNA and by-products. The supernatant containing purified ssDNA was transferred to a fresh microtube as the ssDNA sub-library for the next round of screening.

Absolute quantitative analysis of ssDNA

Quantitative real-time PCR (qPCR) is the most reliable and accurate way of evaluating enrichment. Thus, qPCR was used to monitor the enrichment efficiency in each round. qPCR was performed on the LightCycler 480II (Roche Diagnostics Ltd., Rotkreuz, Switzerland). All reactions were carried out in triplicate in a 96-well microtitre plate. The reaction mixture consisted of 1 μL magnetic selection beads bound to ssDNA, 10 μL SYBR Green I master mix (Mannheim, Germany), 1 μL of mixed primers (each primer 5 pmol/μL), and 8 μL nuclease-free water. The reaction conditions included pre-incubation at 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 seconds, 59 °C for 10 seconds, and 72 °C for 10 seconds. A melting gradient was developed over the range from 65 °C to 95 °C, with increments of 1 °C/s, followed by heating to 97 °C, with two acquisitions per degree increase. Nuclease-free water was used as a negative control.

The ssDNA library was employed as a positive control to generate standard curves used in order to calculate copy numbers. Each of the ssDNA standards was serially diluted from 1.13×10^{12} to 1.13×10^{1} copies/μL in nuclease-free water. An absolute quantitative assay was performed as described above for each dilution. LightCycler 480 software V1.5.1.62 was used for the data analysis, which included the determination of crossing points by the fit point method to produce standard curves. The standard curves were then used to determine the copy number of ssDNA on magnetic selection beads.

Sequencing of ssDNA aptamers

After 14 rounds of selection, the purified ssDNA was sequenced using a Personal Genome Machine (PGM) system. The Ion 314 Chip detects polymerase-driven base incorporation and translates this information into digital form. Each Ion 314 Chip contains 120×10^{6} reaction wells (PostLight semiconductor-based), combined with an Ion Sequencing 200 kit which can generate 400,000-550,000 reads per run, and 30-100 Mb of sequence data.

In this study, emulsion PCR was carried out using Ion OneTouch™ 200 Template kit v2 DL according to the manufacturer's instructions. Briefly, a dsDNA amplicon was amplified from the ssDNA library with two groups of primers, which included the same conserved sequences and different adapter sequences at the 5' and 3' end (CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG, CCT CTC TAT GGG CAG TCG GTG AT, CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG and CCT CTC TAT GGG CAG TCG GTG AT, respectively). Using this primer strategy, the ssDNA template could be amplified and sequenced equally in both the forward and reverse directions. The amplicon libraries were sequenced using a 314 chip Ion Torrent PGM system and the Ion Sequencing 200 kit. Following sequencing, individual sequence reads were filtered by PGM software to remove low quality and polyclonal sequences. All sequencing and data analyses were performed by Life Technologies (Shanghai, China). All kits and software used in this process were designed by Life Technologies.

Blocking the antigen-antibody reaction

A haemagglutination inhibition assay was performed to screen ssDNA aptamers with biological activity for blocking antigen-antibody reactions. In this assay, ssDNA aptamers bind to anti-D and act as neutralising ligands thereby preventing the binding of anti-D to RhD antigens on the surface of erythrocytes. Briefly, 1 μL anti-D with a titre of 128 and 10 pmol ssDNA aptamer were added to 100 μL normal saline and incubated at 37 °C for 30 minutes. Further to this, 100 μL of 2% RhD positive erythrocyte suspension were added and co-incubated at 37 °C for 30 minutes. After washing the erythrocytes with saline three times, 100 μL of FITC-conjugated goat anti-human IgG ($F_{(ab')2}$ fragment, 1:100 dilution) were added and incubated at 37 °C for 30 minutes. After washing the erythrocytes with saline three times, the erythrocyte fluorescence intensity was detected by a BD FACSCanto™ II flow cytometer (BD Biosciences, Piscataway, NJ, USA). Normal saline and ssDNA aptamer candidates were used as the negative controls (100% blocking), and normal saline replacing ssDNA aptamer candidates was used as the positive control (0% blocking). All reactions were run in triplicate in 96-well microtitre plates. ssDNA aptamers which led to reduced detection of FITC intensity were considered to be bio-active candidates.

Analysis of aptamer secondary structures

Secondary structures were calculated using the Mfold web server of M. Zuker[24], and the calculated free energies in Mfold are based on the work by SantaLucia[25]. Linear ssDNA folding was carried out at 37 °C, with 1.0 M Na^{+} in the absence of Mg^{2+}.

Assays of aptamer affinity and specificity

Affinity was determined by saturation curves of candidate ssDNA aptamers binding to anti-D-coated

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microspheres. The procedure was as follows: Alexa Fluor 488-labelled candidate ssDNA aptamers were added to 50 μL anti-D-coated microspheres at concentrations of 50, 100, 200, 400 and 800 nM, and incubated at 37 °C for 30 minutes. After washing the microspheres with normal saline three times, microsphere fluorescence intensity was detected by flow cytometry. Blank controls were produced in a similar manner. All reactions were run in triplicate in 96-well microtitre plates. To calculate the dissociation constant (K_d), the change of fluorescence intensity (y-axis) was plotted against increasing ssDNA aptamer concentrations (x-axis). Data points were fitted by non-linear regression analysis based on a one-site specific binding model in GraphPad Prims V6 software (San Diego, USA). The equation for the calculation was as follows:

$$Y = B\text{max}*X/Kd+X$$

where Bmax is the degree of saturation (Bmax is the maximum specific binding in the same units as Y).

To analyse the specificity of the candidate ssDNA aptamers, microspheres coated with common blood group antibodies (anti-A, anti-B and anti-Fy^a), the main reactive immunoglobulins in humans (IgG and IgM whole molecule), and the main reactive protein (protein A and BSA) were used. Alexa Fluor 488-labelled candidate ssDNA aptamers (10 pmol) were added to 50 μL of different antibody- or protein-coated microspheres, and incubated at 37 °C for 30 minutes. After washing microspheres with normal saline three times, bead fluorescence intensity was detected by flow cytometry. Blank controls were produced in a similar manner. All reactions were run in triplicate in 96-well microtitre plates. The fluorescence intensity of coated beads was then compared with that of anti-D-coated microspheres.

Dose-response analyses

To confirm the bio-activity of selected ssDNA aptamers, erythrocyte agglutination induced by anti-D/RhD ligation was assayed. Briefly, 100 μL anti-D-coated microspheres were incubated with ssDNA aptamers at concentrations of 0, 25, 50, 100, and 200 pmol at 37 °C for 30 minutes before the addition of 50 μL of 5% RhD-positive erythrocytes. The resulting mixture was incubated at 37 °C for 30 minutes and then 100 μL of monospecific anti-human IgG were added and centrifuged at 1,000 *g* for 15 seconds. Erythrocytes were re-suspended by gentle shaking and the degree of agglutination was observed under an Olympus BX43 microscope (Olympus, Tokyo, Japan)[26]. RhD-negative erythrocytes were used as negative controls.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics v22 software (Chicago, IL, USA). Data are expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) with 95% confidence. If homogeneity was achieved, a Tukey honest significant difference test for original values was performed for comparison of multiple groups, otherwise, a Dunnett T3 test was applied. A value of $p<0.05$ was considered to be statistically significant.

Results and discussion

***In vitro* enrichment of ssDNA aptamers targeting anti-D**

To increase the selectivity for candidate aptamers specifically targeting anti-D, the concentration of ssDNA sub-library, magnetic selection beads and selection incubation time were gradually decreased with increasing rounds (see Online Supplementary Table SI). In each round, an absolute quantitative assay for ssDNA was performed in order to measure the degrees of enrichment. Online Supplementary Figure S1 shows that the copy numbers of candidate aptamers increased significantly from 1.98×10^5 copies/μL to 5.40×10^8 copies/μL in the first three rounds. From rounds 4 to 11, although the input amount of ssDNA sub-library and magnetic beads decreased, the order of magnitude of copy number was maintained at the same level of 10^9, indicating an enrichment of aptamers able to bind to anti-D. The selection process from rounds 12 to 14 showed that the duration of selection incubation played a key role in enrichment. Prolonged incubation improved ssDNA aptamer enrichment, but may have also resulted in non-specific binding. Through instant selection, an approximately 2,000-fold enrichment (14^{th} round/1^{st} round) was achieved, as estimated by ssDNA quantitative assays following screening.

The purity of the ssDNA sub-library used for each selection round was evaluated by agarose gel electrophoresis and band intensity was analysed with Image J software. Online Supplementary Figure S2 shows that all dsDNA and by-products were eliminated from the asymmetric PCR mixture. ssDNA concentration was determined by Qubit fluorescent quantitative detection (Qubit ssDNA assay kit, Life Technologies, Carlsbad, CA, USA), with the concentration of purified ssDNA calculated to be 19.2 μg/mL (710 mM) by the 14^{th} round.

ssDNA aptamer sequencing

Many novel ssDNA aptamers with strong binding forces can be produced through the mechanisms of recombination jumping, slippage and bias during the PCR amplification process[27,28]. Emulsion PCR used

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in ssDNA sequencing is a powerful technology for amplifying complex ssDNA libraries in a water-oil emulsion[29,30]. This technology increased the opportunity for screening out ssDNA aptamers according to certain criteria. Analysis of the random sequence length of candidate aptamers targeting anti-D indicated that they were not only composed of 40 nt but also other base pair lengths. The random sequence composed of 35-45 nt accounting for 85.81% of total copies was identified as the advantage sequence (Online Supplementary Figure S3A). In addition, candidate ssDNA aptamers carried different adaptors, as shown in Online Supplementary Figure S3B. In this work, the advantage ssDNA aptamer sequences (copies >1,000, length 35~45 nt) in the screened ssDNA motif library were selected and synthesised to validate their biological activity for neutralising anti-D (Online Supplementary Table SII).

Biological activity of candidate ssDNA aptamers and their secondary structures

In order to examine the biological activity of selected ssDNA aptamers as candidates for blocking RhD antigen-anti-D binding, the blocking antigen-antibody reaction was performed. One-way ANOVA results showed that four of 12 candidate ssDNA aptamers resulted in reductions in fluorescence intensity of anti-D binding to RhD antigens (F[12, 26]=137.37, p<0.01). ssDNA ID 2, 3, 7 and 8 reduced the fluorescence intensity of anti-D binding to RhD antigens to a statistically significant degree (p<0.01) as shown in Online Supplementary Figure S4. Therefore, four ssDNA aptamers with the ability to block anti-D binding to RhD antigens were identified from the candidates selected by screening (Online Supplementary Table SIII and Online Supplementary Figure S4).

The stability of the secondary structure of ssDNA aptamers is determined by base-pairing interactions such as Watson-Crick base-pairing and base-stacking interactions. Secondary structure can be predicted through mathematical and computer-based methods[31-33]. Online Supplementary Figure S4 shows that ssDNA aptamers targeted to anti-D possessed common structural motifs, e.g. multi-branched loops (A), stem and loop structures (B), purine-purine mismatches (C), and bulge loops (D). The ssDNA aptamers that were positive in the screening contained two- to three-branched loop structures, comprising a stem and loop. The stem was formed of complementary base pairs. Double helix regions and loop structures were formed by unpaired bases at the ends of the complementary sequence regions. Purine-purine mismatches were also present. There were two continuous purine-purine mismatches (A:A, G:G) in the middle area of the complementary sequence region in one branched loop structure in ssDNA aptamers 2, 3 and 7. A bulge loop consisting of five bases in ssDNA aptamer 8 was also demonstrated. In principal, the stability of secondary structures is proportional to the number of hydrogen bonds formed by base pairs and dispersion forces arising from base stacking[34]. Loops lead to a decrease in aptamer stability[35,36], but loop orientation also affects their molecular recognition[37]. Online Supplementary Figure S5 demonstrates the overarching principals of these interactions: (i) higher numbers of branched loop structures result in smaller Gibbs free energy (G) values. For example, ssDNA aptamers 7 and 8 have three branched loops with ΔG of −7.47 and −7.37 Kcal/mol, respectively, while aptamers 2 and 3 with two branched loop structures have a ΔG of −5.74 and −6.16 Kcal/mol, respectively; (ii) with a consistent number of branched structures, increasing base pair number results in smaller Gibbs free energy. For example, aptamer 7 with 14 base pairs has a lower free energy than aptamer 8 with 10 base pairs; (iii) with the same number of base pairs, structures without bulge loops were more stable. For example, although ssDNA aptamers 3 and 7 both possess 14 base pairs, aptamer 7 has no bulge loop.

Specificity of effective ssDNA aptamers

The specificity of effective ssDNA aptamers was determined by examining their binding capacity to microspheres coated with common blood group antibodies of the Chinese population, compared with their capacity to bind to anti-D-coated microspheres (Figure 2). The results show that effective ssDNA aptamers have a similar ability to bind to anti-D coated microspheres (F[3, 8]=2.078, p>0.05). However, only ssDNA aptamer 7 displayed low levels of cross-binding to anti-A or anti-Fy^a antibodies and minimal binding to IgG, IgM or BSA, indicating a high degree of specificity. Aptamers 2 and 8 bound to common blood group antibodies within the Chinese population, except for anti-B (Figure 2A). In contrast, aptamer 3 showed no specificity and was, therefore, excluded from further study. It is worth noting that aptamers 2, 7 and 8 had minimal binding to protein A.

Determination of ssDNA aptamer affinity

The affinity of the three selected specific ssDNA aptamers for anti-D was examined further (Figure 3). The Kd values of the binding of ssDNA aptamers 2, 7 and 8 to anti-D were 51.46±14.90, 543.30±92.59, and 403.70±71.47 nM, respectively, indicating that aptamer 2 had the highest affinity, followed by aptamers 8 and 7.

Dose-dependence of the blocking effect

To investigate the dose-dependent inhibitory effect of ssDNA aptamers and in order to identify the optimal

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concentration of aptamer, a concentration gradient from 25 to 200 pmol of single or combined ssDNA aptamers (2, 7 and 8) was used to neutralise anti-D. In order to observe clear results of the indirect anti-human globulin test, anti-D-coated microspheres were used in this test. Table II shows that there is a relationship between the concentration of ssDNA aptamers and the inhibition of haemagglutination. Our results suggest that the higher

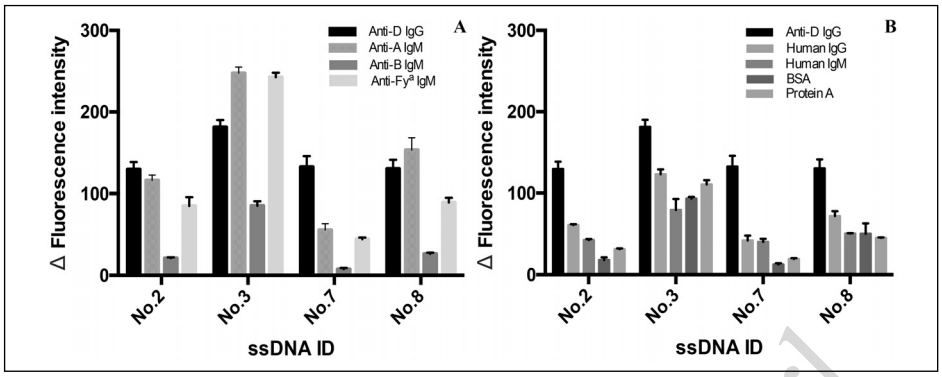


Figure 2 - Verification of the specificity of effective ssDNA aptamers.
ssDNA: single-stranded DNA; IgG: immunoglobulin G; IgM: immunoglobulin M; BSA: bovine serum albumin.

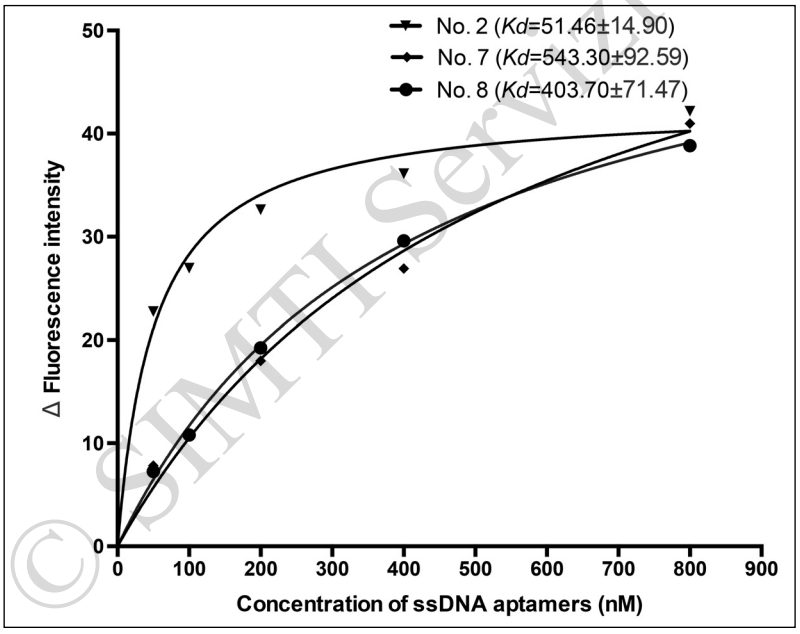


Figure 3 - Binding saturation curve of ssDNA aptamers to anti-D.
ssDNA: single-stranded DNA.

Table II - Dose-dependence of blocking effect on the indirect antihuman globulin test.

ssDNA aptamer ID	ssDNA aptamer concentration			
	25 pmol	*50 pmol*	*100 pmol*	*200 pmol*
2	2+	±	−	−
7	3+	2+	±	−
8	2+	±	−	−
2+7	±	−	−	−
2+8	+	−	−	−
7+8	2+	±	−	−

ssDNA: single-stranded DNA.

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the dose of ssDNA aptamer, the greater the degree of inhibition of haemagglutination. We found that combinations of ssDNA aptamers were more effective than single ssDNA aptamers. At 25 pmol, combinations

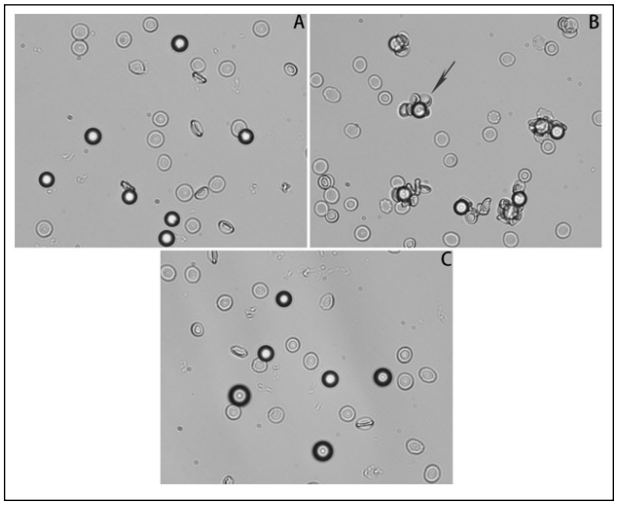


Figure 4 - Microscopic images of the indirect antihuman globulin test.
(A) Negative control. (B) Positive control. (C) Anti-D neutralised by ssDNA aptamers 2 and 7 at 50 pmol. The arrow points to a clot formed by anti-D microspheres and erythrocytes. Magnification 40×10.

of ssDNA aptamers 2 and 7 displayed mixed-field agglutination under microscopic analysis. When the concentration was increased to 50 pmol, agglutination was completely abolished (Figure 4).

Conclusions

Our study is the first to demonstrate that ssDNA aptamers identified and selected via SELEX technology have the ability to neutralise anti-D allo-antibodies. All identified aptamers showed the ability to block RhD/anti-D reactions, and the effect was significantly increased when two of the three ssDNA aptamers were administered concurrently. Through the combined use of ssDNA aptamers 2 and 7, anti-D could be completely neutralised even at low concentrations of the aptamers (each 50 pmol). Our results show a promising strategy for the use of ssDNA aptamers as a potential clinical treatment for delayed haemolytic transfusion reactions and haemolytic disease of the foetus and newborn.

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Authorship contributions

YZ participated in the design of the study and performed the ssDNA SELEX, verified the effects of the aptamers and drafted the manuscript. FW carried out the immunoassays. MW participated in the analysis of aptamer secondary structures. NZ participated in studies of the blocking effect of the aptamers. HZ performed the statistical analysis. HX conceived the study and participated in its design. All Authors read and approved the final manuscript.

The Authors declare no conflicts of interest.

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