

Single-strained DNA aptamers mask RhD antigenic epitopes on human RhD⁺ red blood cells to escape alloanti-RhD immunological recognition

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Abstract. Rhesus D⁻ (RhD⁻) individuals should receive Rh-matched blood to prevent hemolytic anemia. However, there is a shortage of RhD⁻ blood. This study aimed to generate RhD antigen-specific single-stranded DNA (ssDNA) aptamers, and test their efficacy in masking RhD antigens on RhD⁺ red blood cells (RBCs) to prevent their immunoreactivity *in vitro*. In the present study, ssDNA aptamer candidates were synthesized as a central randomized sequence of 40 nucleotides (nt) flanked by 21-nt primer hybridization sequences. The functional aptamers were screened using the cell-based systematic evolution of ligands by exponential enrichment technique and RhD⁺ RBCs. Two bioactive ssDNA aptamers significantly inhibited the binding of an anti-RhD antibody to RhD⁺ RBCs and bound to RhD antigens with high affinity (dissociation constant values of 580.5±142.0 and 737.7±161.8 nM, respectively). Furthermore, treatment with both ssDNA aptamers (500 pmol) effectively masked RhD antigens on 4,000,000 RhD⁺ RBCs to prevent human anti-RhD alloantibody-mediated binding, RBC agglutination and monocyte recognition *in vitro*. Collectively, such data suggested that these ssDNA aptamers may be feasible for masking RhD antigens on RBCs, and thus valuable for

prevention or at least amelioration of RhD⁺-related hemolytic anemia in RhD⁻ individuals.

Introduction

The rhesus (Rh) blood group system is crucial for the safety of transfusion medicine for RhD⁻ recipients (1). The Rh system is the largest and most polymorphic blood group system containing 55 antigens, of which the D antigen is the most clinically relevant (2,3). The RhD antigen has greater immunogenicity than virtually all other red blood cell (RBC) antigens (4). A previous study reported that 30 μl of RhD⁺ RBCs can induce potent humoral responses in a RhD⁻ recipient (5). To ensure transfusion safety, RhD⁻ individuals should receive RhD⁻ donor blood. However, there is a clear shortage of RhD⁻ blood, and RhD⁻ individuals have a frequency of only 0.1-0.4% in China (6-8). This, together with an increase in the demand for clinical transfusion, increases the risk for RhD⁻ individuals, particularly in emergency conditions (9-11). Transfusion with RhD⁺ blood to RhD⁻ individuals can be life-saving in emergencies, but may cause hemolytic anemia induced by endogenous RhD-specific alloantibodies (12,13). Therefore, the development of new therapies to prevent RhD-related hemolytic anemia will be important in the management of blood transfusion for RhD⁻ individuals.

Cell-based systematic evolution of ligands by exponential enrichment (cell-SELEX) has been demonstrated to be an effective strategy in generating universal RBCs with shielded antigens (14,15). Aptamers are short single-stranded DNA (ssDNA) or RNA oligonucleotides that can bind to target molecules with high affinity and specificity (15). Aptamers can be generally screened from a large nucleic acid library containing random sequences through the iterative *in vitro* selection and amplification process of SELEX (16). Aptamers are also called 'chemical antibodies' and have been successfully used as a targeted therapy in clinical treatment (17,18). In theory, specific aptamers targeting RhD antigen epitopes can be obtained by using RhD⁺ RBCs as target cells to screen a nucleic acid library through appropriate cell-SELEX procedures. However,

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Abbreviations: Rh, rhesus; ssDNA, single-stranded DNA; PGM, personal genome machine; RBC, red blood cell; qPCR, quantitative PCR; FI, fluorescence intensity; IAT, indirect agglutination test; MMA, monocyte monolayer assay; PBMCs, peripheral blood mononuclear cells

Key words: ssDNA, aptamer, RhD alloantibody, hemolytic transfusion reaction, immune escape

there is no available aptamer to mask RhD antigen epitopes for transfusion medicine in the clinic.

In the present study, the hypothesis that ssDNA aptamers could be screened using RhD⁺ RBC-SELEX procedures was tested. It was also assessed whether these RhD-specific ssDNA aptamers could mask RhD antigen epitopes on RBCs to prevent the binding of RhD-specific alloantibodies and eliminate the immunoreactivity of RhD⁺ RBCs.

Materials and methods

RBCs. O-type RhD⁺ RBC samples were obtained from 10 non-parental healthy donors (four females and six males, aged 22-41 years old) from Shenzhen Blood Center in August 2016. RBCs were washed three times with saline and then made into 3% saline suspension of RBCs by adding 50 μ l 3% RBC saline suspension and 50 μ l IgM anti-RhD (cat. no. 20163402337; Shanghai Hemo-Pharmaceutical & Biological) reagent into a clean tube, and the agglutination was observed after centrifugation at 800 x g for 15 sec. The RBCs showed strong agglutination in the presence of anti-RhD IgM. After being washed, the RBCs were adjusted at 4x10⁶ RBCs/ml in citrate-phosphate-dextrose-adenine solution and used as the target cells for cell-SELEX. Similarly, AB-type RhD⁻ (*RHD*⁻/*RHD*⁻) RBC samples obtained from 10 non-parental healthy donors (five females and five males, aged 22-35 years old) were prepared in the same manner as the control cells. All donors provided written consent for this study.

RhD antibodies. The IgG monoclonal anti-RhD antibody was purchased from Shanghai Hemo-Pharmaceutical & Biological (cat. no. 20160725). Human RhD alloantibodies were obtained from the serum of three female donors (named 1-3, aged 27-34 years old), who had fetuses and/or newborns with RhD-associated hemolytic disease at the Shaanxi Institute of Transfusion Medicine in July 2016. The titers of all antibodies were determined by an antibody titration procedure, conducted as previously described, to reduce inter-laboratory variation (19). The monoclonal anti-RhD antibody at 1:128 dilution had a strong agglutination activity with a titer of 512 [1,024 (titer of the highest dilution of serum that gives a reaction), score 109 (sum of scores for all tubes in the titration study)], and RhD alloantibody 1 at 1:8 dilution displayed a strong agglutination activity with a titer of 64 (128, score 80). RhD alloantibody 2 at 1:2 dilution had a titer of 64 (256, score 70), and RhD alloantibody 3 at 1:1 dilution had a titer of 32 (64, score 57).

SELEX ssDNA library and PCR primers. The ssDNA library and primers for PCR were synthesized by Invitrogen; Thermo Fisher Scientific, Inc., and purified by high performance liquid chromatography. Individual ssDNAs in the library contained a central randomized sequence of 40 nucleotides (nt) flanked by 21-nt primer sequences (5'-AGAGACGGACACAGGATGAGC-40nt-CCTTCCCCAAGACAGCATCCA-3'). The selected ssDNA pool was amplified by symmetric PCR using the sequences of biotinylated primers (forward 5'-biotin-AGAGACGGACACAGGATGAGC-3', reverse 5'-biotin-TGGATGCTGTCTTGGGGAAGG-3'). The non-biotinylated excessive

forward primer (5'-AGAGACGGACACAGGATGAGC-3') and partially biotinylated reverse primer (5'-biotin-TGGATGCTGTCTTGGGGAAGG-3') were used in asymmetric PCR at a ratio of 20:1 (20).

Generation and purification of ssDNA sub-libraries. The ssDNA sub-libraries were generated by an indirect purification method, as described previously (20,21). Briefly, the ssDNA library was first selected by the symmetric PCR in a 50- μ l reaction volume containing 10 μ l template, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0), 1.5 mM MgCl₂, 400 μ M each dNTP, 2.5 U Taq DNA polymerase (Promega Corporation), and 50 pmol each biotinylated primer. The PCR was performed at 94°C for 5 min, followed by 11 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, followed by 72°C for 5 min. Subsequently, the PCR products were selected by asymmetric PCR in a 20- μ l reaction volume containing 1 μ l symmetric PCR product as the template, 2 μ l forward primer (10 pmol/ μ l), 2 μ l biotinylated reverse primer (0.5 pmol/ μ l; primers at ratio of 20:1), 10 μ l of AmpliTaq Gold Fast PCR master mix (Thermo Fisher Scientific, Inc.). The asymmetric PCR reactions were performed at 96°C for 10 min, then 30 cycles of 96°C for 3 sec, 59°C for 3 sec and 68°C for 3 sec, followed by 72°C for 10 sec. The asymmetric PCR products were selected by streptavidin-coated magnetic beads (Z5482, Promega Corporation) to eliminate biotinylated double-stranded DNA and by-products. Briefly, streptavidin-coated magnetic beads (0.48 mg) were washed three times with PBS-Tween 20 (pH 7.4, with 0.02% Tween-20) and were captured using a magnetic stand after each wash. Asymmetric PCR products (320 μ l) were added to washed beads and incubated for 20 min at 25°C. After magnetic separation, the supernatants were collected. The unbound ssDNAs were precipitated with sodium acetate (3 M, pH 5.5) and 70% ethanol. Finally, the ssDNAs were dissolved in normal saline as ssDNA sub-libraries for the next round of screening (Table I).

In vitro cell-SELEX. The ssDNA sub-libraries in normal saline were further screened by the cell-SELEX strategy described previously, with some modifications (Fig. 1) (22,23). Briefly, the ssDNA sub-libraries were heat-denatured at 95°C for 5 min, snap-cooled and reacted with RhD⁻ RBCs in the presence of 0.1 mg/ml salmon sperm DNA (Thermo Fisher Scientific, Inc.) for 30 min at 25°C. After centrifugation at 1,000 x g for 1 min at 25°C, the supernatants were recovered and incubated with RhD⁺ RBCs for 30 min at 25°C. The RBCs were magnetized for 2 min using 200 μ l MagneLys solution (Diagast) following which they were washed with normal saline five times, and then the ssDNAs bound on the magnetized RBCs were extracted. These were used as templates for PCR amplification in order to generate ssDNA sub-libraries using the Dynabeads SILANE viral NA kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. To increase the stringency of selection, the concentrations of ssDNAs and positive selection time were gradually reduced throughout the selection process (Table I).

Enrichment analysis. The enrichment efficiency of ssDNAs was determined by quantitative PCR (qPCR) using a LightCycler 480II (Roche Diagnostics). The reaction mixtures

Table I. Selection conditions for the cell-systematic evolution of ligands by exponential enrichment process.

Round	ssDNA (pmol)	RhD ⁻ cells (x10 ⁶)	Counter selection time (min)	RhD ⁺ cells (x10 ⁶)	Positive selection time (min)
1	2,750	2	30	2	30
2	1,000	4	30	4	30
3-5	500	4	30	4	30
6-8	100	2	30	4	30
9-11	50	2	30	2	30
12-13	50	1	30	2	10
14	50	0	0	2	5

ssDNA, single-stranded DNA; Rh, Rhesus.

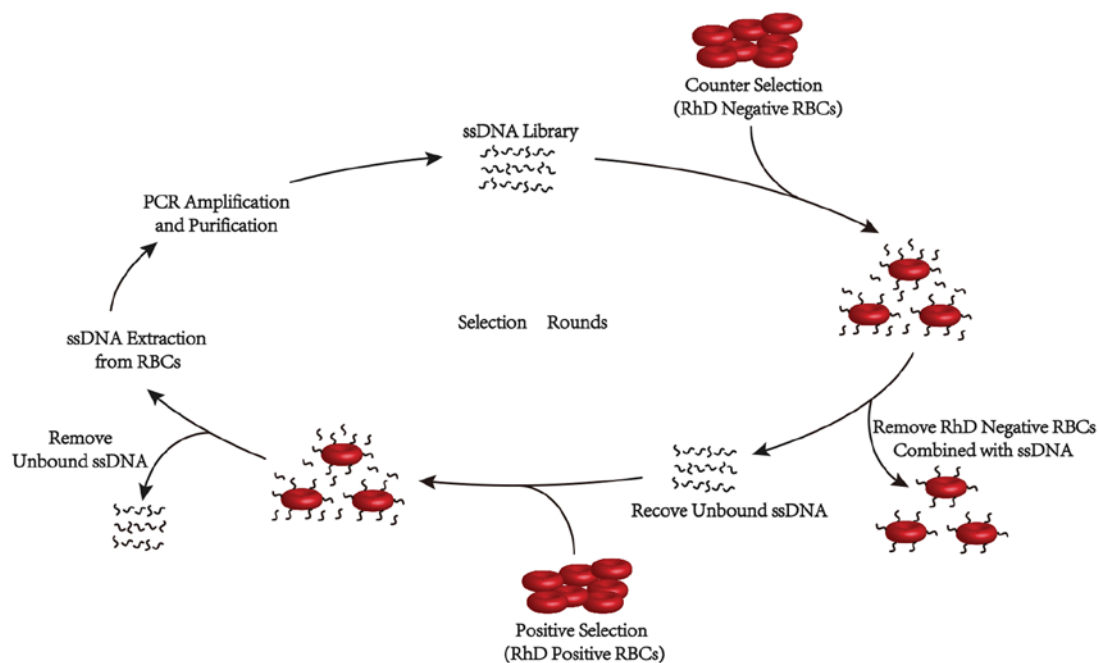


Figure 1. Scheme of *in vitro* selection of ssDNA aptamers by the cell-SELEX process. The SELEX process consists of three main steps for each selection round: i) Counter selection by incubating the ssDNA library with RhD⁻ RBCs; ii) positive selection by incubating the recovered unbound ssDNAs with RhD⁺ RBCs; and iii) recovery of bound ssDNA from RhD⁺ RBCs and re-amplification of recovered ssDNAs. ssDNA, single-stranded DNA; SELEX, systematic evolution of ligands by exponential enrichment; Rh, Rhesus; RBCs, red blood cells.

consisted of 1 μ l magnetic ssDNA, 10 μ l SYBR Green I master mix (Roche Diagnostics GmbH), 1 μ l mixed primers (forward, 5'-AGAGACGGACACAGGATGAGC-3' and reverse, 5'-TGGATGCTGTCTTGGGGAAGG-3'; each primer 5 pmol/ μ l) and 8 μ l nuclease-free water. The reactions were performed at 95°C for 5 min and subjected to 45 cycles of 95°C for 10 sec, 61°C for 10 sec, and 72°C for 10 sec. Nuclease-free water served as a negative control. Each test was repeated three times to verify the data repeatability and the mean value was analyzed using LightCycler 480 software V1.5.1.62 (Roche Diagnostics), and the copy number of ssDNA was determined by comparing C_t values with those from the standard curves.

Sequencing of ssDNA aptamers. After 14 rounds of selections, the purified ssDNAs were sequenced, as described previously (21). In brief, the ssDNA was amplified by PCR, as

described above, in the presence of the adapter sequences (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3', 5'-CCCTCTATGGGCAGTCGGTGAT-3', 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3' and 5'-CCTCTCTATGGGCAGTCGGTGAT-3') and sequenced by the Personal Genome Machine (PGM) System. Individual sequence reads were filtered by PGM (Torrent suite software v3.0) to remove low-quality (<10 copies) and polyclonal sequences and analyzed by Ion reporter server system software 5.0 (Thermo Fisher Scientific, Inc.). The remaining reads were clustered, and the most abundant sequences were chemically synthesized for further characterization. All sequencing was performed and analyzed by Thermo Fisher Scientific, Inc.

Immunofluorescence. The masking efficacy and affinity of the aptamer candidates binding to RhD antigen epitopes

were determined by direct and indirect immunofluorescence. Briefly, RhD⁺ RBCs (4x10⁶ cells/well) were blocked in triplicate with ssDNAs (500 pmol, 100 μ l saline) in 96-well plates at 25°C for 60 min and incubated with 50 μ l standardized monoclonal anti-RhD antibody (1:128) for 30 min at 25°C. After being washed, the antibody bound to RBCs was detected with FITC-conjugated goat anti-mouse IgG F(ab')₂ fragment (1:2,000; cat. no. AB616780; Bio-Rad Laboratories, Inc.) for 30 min at 25°C. Subsequently, the fluorescent signals in RBCs were detected analyzed by Attune Nxt acoustic focusing cytometry and Attune software v1.1.0 (Thermo Fisher Scientific, Inc.). RhD⁺ RBCs incubated with FITC-conjugated goat anti-mouse IgG were used as the blank controls, and normal saline replacing ssDNA aptamer candidates served as the positive control. Data were expressed as fluorescence intensity (FI) using the following formula: FI=(test group RBCs)-FI (blank control). Individual ssDNA aptamers, which led to reduced detection of FI compared with the positive control, were considered to be bioactive candidates.

The affinity of each ssDNA candidate binding to RhD antigens was determined by direct immunofluorescence. Individual ssDNA candidates were labeled with Alexa Fluor[®] 488, these were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. RhD⁺ RBCs (2% suspension, 100 μ l/well) were stained with different concentrations (200-1,200 nM) of Alexa Fluor 488-labeled ssDNA at 37°C for 60 min. After being washed, the cells were analyzed by Attune Nxt acoustic focusing cytometry and Attune software v1.1.0 (Thermo Fisher Scientific, Inc.). The dissociation constant (K_d) was analyzed by plotting fluorescence intensity (y-axis) against ssDNA aptamer concentrations (x-axis) using GraphPad Prism V6 software (GraphPad Software, Inc.). The equation for the calculation was $Y = B_{max} \times X / K_d + X$ (B_{max} is the degree of saturation with maximum binding in the same units as Y).

Shielding RhD antigens. The ability of ssDNA aptamers to mask RhD antigens was determined by indirect immunofluorescence and the indirect agglutination test (IAT). The procedure of indirect immunofluorescence was performed, similar to that described above, except for using varying concentrations (0, 200, 300, 400 or 500 pmol) of each ssDNA aptamer.

An IAT was performed, as described previously (24). Briefly, RhD⁺ RBCs (4x10⁶ cells/well) were incubated with different concentrations of ssDNA aptamers in 100 μ l saline for 60 min and incubated with 100 μ l standardized human anti-RhD alloantibodies at 37°C for 30 min. After being washed, 100 μ l anti-globulin (cat. no. 20153401143; Shanghai Hemo-Pharmaceutical & Biological) was added to the dry RBCs and centrifuged immediately at 1,000 x g for 15 sec at 25°C. The formed RBC clusters were re-suspended by gentle shaking and the degree of agglutination was evaluated with a Axioscope A1 microscope (Zeiss AG), using magnification, x10). The reactive system without ssDNA aptamer or RBC served as positive and negative controls, respectively.

Monocyte monolayer assay (MMA). A MMA was performed, as previously described (25). In brief, peripheral blood mononuclear cells (PBMCs) from 6 healthy donors (four females and two males, aged 19-35 years old) were isolated by

Ficoll-Hypaque density gradients (500 x g for 25 min at 25°C) and used as the effectors. After being washed, the PBMCs from the 6 donors were mixed and adjusted to a concentration of 1x10⁶ cells/ml. RhD⁺ RBCs were used as the targets. Simultaneously, RhD⁺ RBCs were incubated with 2-fold volumes of saline or standardized human anti-RhD antibodies at 37°C for 30 min as the un-sensitized and sensitized RBCs, respectively. Some RhD⁺ RBCs were pre-incubated with different concentrations (200-500 pmol) of ssDNA aptamers for 30 min at 25°C and sensitized with standardized human anti-RhD antibodies as the experimental RBCs. After being washed, the RBCs were adjusted to a concentration of 1x10⁷ cells/ml. Subsequently, the PBMC suspension (50 μ l) was cultured in RPMI 1640 (Corning Inc.) with 10% FBS (Corning Inc.) on 8-well Lab-Tek[™] II chamber slides (Thermo Fisher Scientific, Inc.) for 60 min at 37°C in 5% CO₂, washed, and co-cultured in triplicate with 100 μ l of sensitized, control un-sensitized or experimental RhD⁺ RBCs for 90 min in 5% CO₂ at 37°C. The slides were washed, air-dried, stained with Wright-Giemsa for 5 min at room temperature, and images were captured using a light microscope (magnification, x40), at least 500 monocytes in each sample were examined under multiple fields. The number of RBC-bound monocytes in individual samples was counted, and at least 500 monocytes in each sample were examined in a blinded manner. Based on reactivity of un-sensitized RBCs, 3% of monocytes with RBC binding was considered a positive MMA result (26).

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics V22 software (IBM Corp.). Each experiment was repeated three times and data are expressed as mean \pm SD. The difference among groups was analyzed by ANOVA with 95% confidence and post hoc Tukey's test. Statistical significance was defined as P<0.05.

Results

Enrichment and sequencing of ssDNA aptamer candidates. To examine the enrichment efficacy of *in vitro* ssDNA aptamer candidates targeting RhD antigens in each cell-SELEX round, the ssDNA copies were quantified by qPCR. The qPCR results showed that the copy numbers of ssDNA aptamer candidates significantly increased near 1,000 folds from 5.43 \pm 0.34x10⁵ copies/ μ l in the first round to 5.01 \pm 0.22x10⁸ copies/ μ l in the last round (P<0.001; data not shown). Sequencing analysis indicated 12 dominant ssDNA aptamer sequences (copies >1,000, random sequence length at 40 bp) after 14 rounds of the ssDNA motif library. The sequences of the most abundant ssDNA aptamers were selected and synthesized.

ssDNA aptamer candidates have the ability to shield RhD antigens. The ability of ssDNA aptamer candidates to shield anti-RhD RhD antigen-specific immune recognition was evaluated by indirect immunofluorescence. The results exhibited that 2 (termed No. 1 and 2) out of 12 advanced ssDNA aptamer candidates significantly decreased the fluorescence intensity of monoclonal anti-RhD binding to RhD antigens (P<0.001; Fig. 2). The central sequences of 40 nt between the upstream and downstream primers were 5'-GGCCTGGTCTGTTAG

Table II. Sequences of variable regions in the advanced ssDNA aptamer candidates.

No.	Variable region sequences (5'→3')	nt
1 ^a	GGCCTGGTCTGTAGCCGGGTAGCAGCCCCGGCACCTATT	40
2 ^a	GGGTAGCAGCCCCGCGGAGGGTCGGCTATAAGAACCAAGA	40
3	CTATCCCCACGTCACCTTTTCCCGTAGGTTGGACTCGACC	40
4	GGTGCAGGGGGGTCGGAGAAGAGGTTGAGGGGAGAGGGGT	40
5	ACGGCCTCTGTATAATGCTGGCCTTGACGCTTGTCCTTG	40
6	ACGGCCTCTGTACAATGCTGGCCTTGACGCTTGTCCTTG	40
7	TACACCAATCTCCCCCTACATTCTCCACCAGCACTCCA	40
8	GGGTAGCAGCCCCGCGGAGGGTCGGCTATAAGAAGTAGGA	40
9	GGGTAGCAGCCCCGCGGAGGGTCGGCTATAAGAACCAGGA	40
10	GGGTAGCAGCCCCGCGGAGGGTCAGCTATAAGAACCAGGA	40
11	GGGTAGCAGCCCCGCGGAGGGTCGGCTATAGGAACCAGGA	40
12	CTATCCCCACGTCACCTTTTCCCGTAGGCTGGACTCGACC	40

^assDNA motif with binding Rhesus D antigen-determining regions to block antibody recognition. ssDNA, single-stranded DNA; Rh, Rhesus; nt, nucleotides.

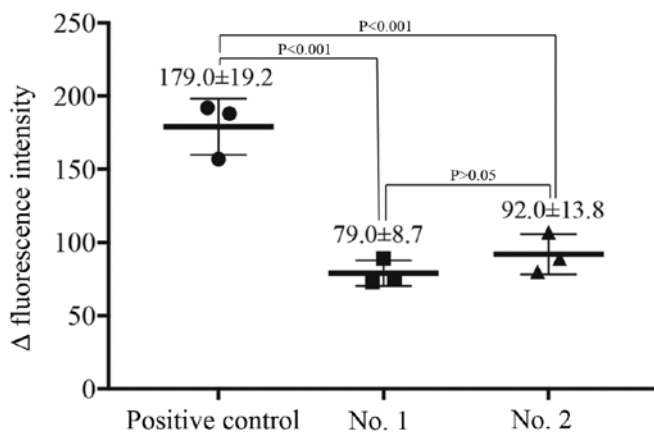


Figure 2. Indirect immunofluorescence analysis of the effect of ssDNA aptamers on immunorecognition of RhD antigens. RhD⁺ red blood cells were treated in triplicate with a vehicle (positive control), No. 1 and 2 ssDNA aptamers, respectively, following which cells were stained with monoclonal anti-RhD antibody and FITC-conjugated goat anti-mouse IgG. The fluorescence intensity in each group of cells was determined by flow cytometry. Data are expressed as the mean ± SD of each group from three separate experiments. ssDNA, single-stranded DNA; Rh, Rhesus.

CCGGGTAGCAGCCCCGGCACCTATT-3' and 5'-GGGTAGCAGCCCCGCGGAGGGTCGGCTATAAGAACCAAGA-3', respectively (Table II). The aim of further direct immunofluorescence was to evaluate the affinity of the two selected specific ssDNA aptamers, it was revealed that treatment with different concentrations of ssDNA aptamers increased the fluorescence intensity in a dose-dependent manner, and the No. 1 and 2 ssDNA aptamers binding to RhD antigens had K_d values 580.5±142.0 and 737.7±161.8 nM, respectively (Fig. 3).

ssDNA aptamers mask RhD antigens for their immunoreactivity. Due to relatively small molecule of ssDNA, combination of ≥2 ssDNA aptamers often has superior efficacy in antigen shielding (21,27). It was observed that treatment with both

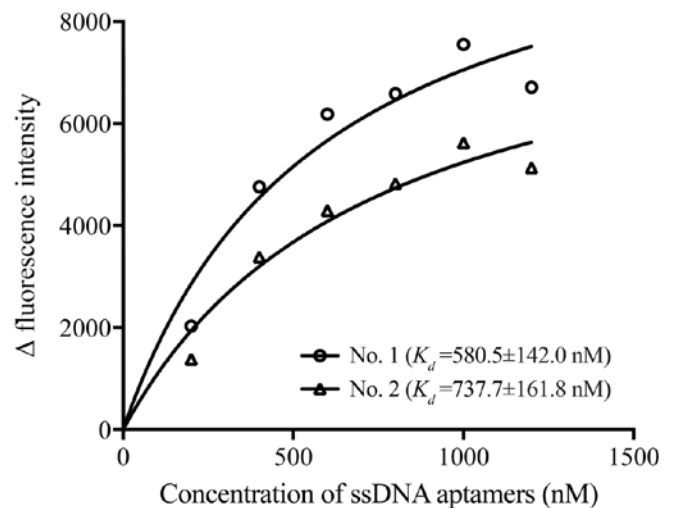


Figure 3. Binding dynamics of ssDNA aptamers to RhD⁺ RBCs. RhD⁺ RBCs (2% suspension, 100 μl/well) were stained in triplicate with different concentrations (200-1,200 nM) of Alexa Fluor 488-labeled each ssDNA at 37°C for 60 min. After being washed, the cells were analyzed by flow cytometry. The K_d was analyzed. Data are plotted by fluorescence intensity (y-axis) against ssDNA aptamer concentrations (x-axis) using GraphPad Prism V6 software. Experiments were performed three times, and data is a mean of the triplicates. ssDNA, single-stranded DNA; Rh, Rhesus; RBCs, red blood cells; K_d , dissociation constant.

No. 1 and 2 ssDNA aptamers (200-500 pmol each) significantly decreased the fluorescent signals of anti-RhD binding to RhD⁺ RBCs in a dose-dependent manner (Fig. 4A). Treatment with both ssDNA aptamers at 500 pmol blocked the fluorescence intensity of anti-RhD (P<0.001 vs. positive control, P>0.05 vs. blank). A similar pattern of antigen shielding ability of ssDNA aptamers was observed by IAT. While positive control RBCs (without aptamer treatment) display varying sizes of agglutinative clusters (Fig. 4B), treatment with 400 pmol ssDNA aptamers resulted in few RBC clusters (data not shown), and treatment with 500 pmol completely prevented the formation

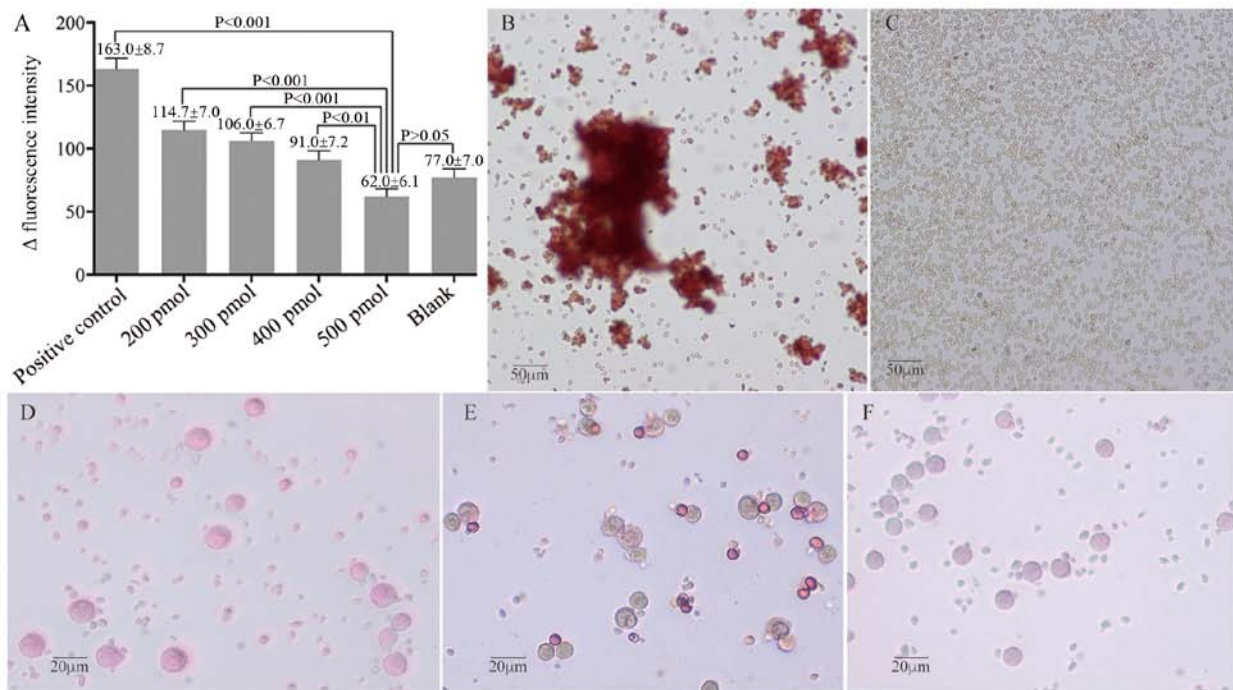


Figure 4. ssDNA aptamers mask RhD antigens. (A) ssDNA aptamers inhibit the binding of human RhD-specific alloantibodies to RhD⁺ RBCs in a dose-dependent manner. Data are expressed as the mean \pm SD of each group from three separate experiments. Representative images (magnification, $\times 100$) of indirect agglutination tests; (B) positive group (without aptamer treatment) displays RBC agglutination, whereas (C) ssDNA aptamers (No. 1 and 2, both at 500 pmol) inhibit the sensitization of human alloantibodies. (D) Negative control group of MMA. (E) Positive control group of MMA. (F) ssDNA aptamers inhibit the sensitization of human alloantibodies for MMA. ssDNA, single-stranded DNA; Rh, Rhesus; RBCs, red blood cells; MMA, monocyte monolayer assay.

of anti-RhD-mediated RBC agglutination *in vitro* (Fig. 4C). Further MMA analysis revealed that treatment of monocytes with sensitized RhD⁺ RBCs resulted in 19.5% monocyte reactivity, while treatment of monocytes with un-sensitized (2.2%) or experimental (2.5%) RhD⁺ RBCs failed to achieve a positive monocyte reactivity ($>3.0\%$; Fig. 4D-F). Such data indicated that the ssDNA aptamers blocked the sensitization of anti-RhD antibodies, leading to a failure of monocytes to bind and phagocytize RhD⁺ RBCs. Collectively, these data demonstrated that the ssDNA aptamers at an optimal dose effectively masked RhD antigens on RhD⁺ RBCs to prevent their immunoreactivity with anti-RhD antibodies.

Discussion

In the present study, two ssDNA aptamers containing a randomized central sequence of 40 nt flanked by 21-nt primer sequences that have been synthesized and screened by human RhD⁺ RBC-SELEX are reported. These ssDNA aptamers effectively masked human RhD antigen epitopes on RhD⁺ RBCs to prevent the binding of an anti-RhD antibody and human RhD-specific alloantibodies to RhD⁺ RBCs. Conceivably, RhD⁺ RBCs may be modified by these ssDNA aptamers and survive in RhD⁻ recipients even if they have RhD-specific alloantibodies. To the best of our knowledge, this was the first study reporting the generation and biological characterization of RhD-specific ssDNA aptamers. These novel bioactive ssDNA aptamers may be valuable in the modification of RhD⁺ RBCs for transfusion into RhD⁻ recipients, thereby reducing the problem of RhD⁻ blood shortages in emergency situations.

First, two ssDNA aptamers were successfully obtained using the cell-SELEX procedure. Each aptamer achieved a 1,000-fold enrichment, supporting the notion that the cell-SELEX procedure is a powerful tool in screening an ssDNA aptamer library. These two novel ssDNA aptamers had a high binding affinity to RhD⁺ RBCs at a nanomolar concentration (K_d 580.5 \pm 142.0 and 737.7 \pm 161.8 nM for ssDNA aptamers No. 1 and 2, respectively). The affinity to these concentrations indicated that the aptamers have a strong binding force, which is consistent with previous findings (28). However, other advanced ssDNA aptamers did not effectively block the binding of the monoclonal anti-RhD antibody to RhD⁺ RBCs. This may stem from the lack of D-antigen specificity, low affinity or high K_d binding of these ssDNA aptamers.

The IAT can analyze *in vitro* antibody-antigen interactions, and has been used to detect antibody-mediated RBC agglutination that may cause hemolytic reactions following transfusion (29,30). In the current study, it was found that treatment with ssDNA aptamers resulted in a negative IAT. It is possible that ssDNA aptamers may bind to RhD antigen epitopes to prevent the binding of anti-RhD and RhD-specific alloantibodies, thus leading to a failure of these antibodies to interact with their targets on RhD⁺ RBCs. However, a combination of aptamers No. 1 and 2 at 500 pmol each was less effective in masking RhD antigen epitopes than each type of aptamer on the binding of anti-RhD monoclonal antibody to prevent RBC agglutination. This may be due to different specificity characteristics of polyclonal alloantibodies and monoclonal anti-RhD antibody. It may be required for more aptamers to mask D antigen epitopes recognized by polyclonal alloanti-RhD. Furthermore, it was observed that treatment

with ssDNA aptamers (No. 1 and 2 together at 500 pmol each) resulted in a trend towards the immunofluorescence intensity being lower than that of RhD background level. This suggests that the non-specific binding of fluorescent antibodies to RBCs may be reduced when ssDNA is bound to RBCs, although this finding was not significant.

The MMA measures Fc γ receptor-mediated phagocytosis of alloantibody-sensitized RBCs and has been successfully used to predict blood transfusion outcomes and avoid immune destruction of antibody-sensitized RBCs (31-33). In the present study, it was found that while co-culture of monocytes with sensitized RhD⁺ RBCs resulted in a rate of 19.5% monocyte reactivity, co-culture of monocytes with RhD⁺ RBCs that had been pre-treated with ssDNA aptamers greatly reduced the monocyte reactivity rate to 2.5%, which was lower than the negative control. This indicated that treatment with both No. 1 and 2 ssDNA aptamers at 500 pmol each completely masked the RhD antigen epitopes recognized by RhD-specific alloantibodies to prevent the RhD-specific alloantibody-mediated phagocytosis by monocytes. It would be interesting to further investigate the safety and therapeutic effect of these ssDNA aptamers, and their stability and immunogenicity *in vivo*. If successful, the RhD-specific ssDNA aptamers may be applicable *in vivo* to prevent, or at least ameliorate, hemolytic reactions in RhD⁻ patients, who have fatal blood loss and require RhD⁺ blood due to the shortage of RhD⁻ blood.

In the present study, RhD-specific ssDNA aptamers were successfully screened using the RhD⁺ RBC-SELEX procedure, and these aptamers effectively masked the RhD antigen epitopes on RhD⁺ RBCs to prevent the binding of anti-RhD and human RhD-specific alloantibodies to RhD⁺ RBCs. Potentially, the RhD-specific ssDNA aptamers may be valuable in the modification of RhD⁺ RBCs for transfusion medicine to prevent or ameliorate hemolytic reactions in RhD⁻ patients, who have fatal blood loss and require RhD⁺ blood due to the unavailability of RhD⁻ blood. The approach reported in the present study may provide a potential way of overcoming the problems presented by RhD⁻ RBCs shortages in an emergency.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon a reasonable request.

Authors' contributions

YZ conceptualized the study. HX designed the study. XW, RL and LL performed the experiments and analyzed the data. LW

and HZ analyzed the data. YZ and HZ wrote the manuscript. HX, XW, LW, RL and LL revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shenzhen University General Hospital (Shenzhen, China). All participants signed written informed consent for the publication of their data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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