Spatial- and Valence-Matched Neutralizing DNA Nanostructure Blocks Wild-Type SARS-CoV-2 and Omicron Variant Infection

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Cite This: https://doi.org/10.1021/acsnano.2c06803

ABSTRACT: Natural ligand–receptor interactions that play pivotal roles in biological events are ideal models for design and assembly of artificial recognition molecules. Herein, aiming at the structural characteristics of the spike trimer and infection mechanism of SARS-CoV-2, we have designed a DNA framework-guided spatial-patterned neutralizing aptamer trimer for SARS-CoV-2 neutralization. The ~5.8 nm tetrahedral DNA framework affords precise spatial organization and matched valence as four neutralizing aptamers (MATCH-4), which matches with nanometer precision the topmost surface of SARS-CoV-2 spike trimer, enhancing the interaction between MATCH-4 and spike trimer. Moreover, the DNA framework provides a dimensionally complementary nanoscale barrier to prevent the spike trimer–ACE2 interaction and the conformational transition, thereby inhibiting SARS-CoV-2–host cell fusion and infection. As a result, the spatial- and valence-matched MATCH-4 ensures improved binding affinity and neutralizing activity against SARS-CoV-2 and its varied mutant strains, particularly the current Omicron variant, that are evasive of the majority of existing neutralizing antibodies. In addition, because neutralizing aptamers specific to other targets can be evolved and assembled, the present design has the potential to inhibit other wide-range and emerging pathogens.

KEYWORDS: spatial-patterned interaction; DNA nanotechnology, neutralizing aptamer, SARS-CoV-2 neutralization, Omicron

The outbreak of SARS-CoV-2 and its variants has underscored the need to rapidly generate specific recognition and treatment strategies against novel pathogens. SARS-CoV-2 presents the specific conformational pattern and transition of the spike trimer, which allow SARS-CoV-2 to bind strongly to host receptor ACE2 via the receptor-binding domain (RBD) on the spike protein. Specifically, in uninfected cells, the majority of the spike trimers are in a tightly closed state, and only a minority is in the intrinsically transient open state with one up RBD representing a fusion-prone state. Once the up RBD is trapped by host ACE2, the associated ACE2–RBD complex swings continuously on the topmost surface of the spike trimer to release the constraints imposed on the fusion machinery, thereby promoting virus–cell membrane fusion and viral infection. Therefore, a neutralization strategy that effectively binds to all three RBDs on the spike trimer will block the ACE2–RBD interaction and subsequent conformational transition, thus achieving an ideal therapeutic effect for COVID-19.

Precise and efficient natural recognition is often based on the formation of a complementary pair with a series of noncovalent bonds and the achievement of the proper interfacial contact. For example, homotrimeric tumor necrosis factor recruits three tumor necrosis factor receptors to form a complex for immune responses and inflammation. Inspired by the spatial distribution and valence-matched multivalent ligand–receptor interaction, we engineered a multivalent aptamer through a tetrahedron-conformational DNA holder (MATCH), to achieve a neutralizing reagent trimer for efficient blocking of SARS-CoV-2 infection via binding to three RBDs of the spike trimer.

Received: July 10, 2022
Accepted: September 6, 2022
Neutralizing antibodies show great potential for direct-acting and immediate therapy. However, exact matching of the three-dimensional structure of the spike trimer is difficult to achieve without major costs in yield and quality. In contrast, single-stranded nucleic acids to neutralize SARS-CoV-2, also known as neutralizing aptamers, offer a parallel strategy to rapidly produce potent antiviral reagents for passive immunization. Watson–Crick base pairing results in straightforward programmability of neutralizing aptamers, thus allowing aptamer–target interaction in a quantitative manner by specific shape and precise valence assembly. In addition, aptamers have favorable chemical and biological properties, including small size, high thermostability, low immunogenicity, and deep tissue penetration. Therefore, aptamers have several advantages over neutralizing antibodies in several aspects and can be engineered as a topologically matched neutralizing reagent for SARS-CoV-2 via DNA nanotechnology. Recently we and other groups have discovered several neutralizing aptamers against SARS-CoV-2, in the form of monovalent, bivalent, and spherical nanoparticles, and precisely spatial- and valence-matched neutralization has rarely been realized.

Given that the RBD−RBD distance is \( \sim 5 \) nm on the topmost surface of the spike trimer resembling an equilateral triangle (Scheme 1A), we strategically designed the MATCH architecture as multivalently arranged neutralizing aptamers in a 2D pattern complementary to an equilateral triangle conformation of the spike trimer (Scheme 1B). Specifically, a \( \sim 5.8 \) nm tetrahedral DNA framework was used as the scaffold to orthogonally anchor aptamers targeting RBDs of the spike trimer. The resultant designs are called MATCH-1–4 with precise spatial organization and quantitative aptamer number. Each side of MATCH-4 has three pieces of neutralizing aptamer arranged in the shape of a \( \sim 5.8 \) nm equilateral triangle (Scheme 1C). Compared to a monomeric aptamer, MATCH-4, with a well-matched structure, enables a spatially patterned multivalent interaction capable of binding three RBDs of the spike trimer by three neutralizing aptamers. Moreover, the DNA framework of MATCH-4 also provides a dimensionally complementary nanoscale barrier to prevent the interaction of spike trimer–ACE2 receptor and the conformational transition of the spike trimer, thus further inhibiting SARS-CoV-2–host cell fusion and infection. With the dual blocking MATCH strategy of spatially patterned multivalent aptamer binding and steric hindrance of the DNA nanostructure, MATCH-4 is expected to inhibit SARS-CoV-2 infection with high efficiency and to serve as a model for the development of neutralization mechanisms for possible future pandemics.

**RESULTS/DISCUSSION**

**Characterization of MATCH-n.** To construct MATCH assemblies with a series of valence states, we orthogonally anchored the neutralizing aptamers on the tetrahedral DNA framework according to the quantitative ratio to generate MATCH-1–4 (SI, Tables S1 and S2). The successful assembly of MATCH-1–4 was confirmed by polyacrylamide gel electrophoresis (PAGE) (>75% yield, Figures 1A and S1).

![Figure 1. Characterization of MATCH-n.](https://doi.org/10.1021/acsnano.2c06803)

To further analyze the stoichiometry of MATCH-1–4, we characterized the valence of MATCH-1–4 containing gold-nanoparticle-modified aptamers by transmission electron microscopy (TEM). As shown in Figures 1B and S2, the number of gold nanoparticles attached to aptamers extending from the framework is controllable, indicating the successful quantitative construction of MATCH-1–4. Additionally, cryoelectron microscopy images showed the morphology and size of MATCH-4 (Figure 1C), further confirming the formation of the tetrahedral DNA structure.

**Binding of MATCH-n against SARS-CoV-2 Spike Trimer.** Changes in valence affect the interaction of MATCH-n assembly and SARS-CoV-2 spike trimer (Figure 2A), which will lead to different inhibition effects on virus infection. The \( K_d \) (dissociation constant) values of MATCH-1–4 against SARS-CoV-2 spike trimer vary inversely with the number of aptamers at vertices. MATCH-4 shows the smallest \( K_d \) value of \( \sim 2.4 \) nM (Figure 2B), which is \( \sim 8.2 \)-fold and \( \sim 18.7 \)-fold lower than that of MATCH-1 and free RBD aptamer, respectively, indicating significantly improved bind-

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**Scheme 1. Mechanism of the MATCH strategy for SARS-CoV-2 neutralization.** (A) Diagram of the trimetric spike protein (PDB code 7KD1). The three RBDs are depicted in gray, pink, and green, respectively. The representative amino acid residues involved in ACE2 binding are indicated in red. (B) A tetrahedral DNA framework based on four neutralizing aptamers (MATCH-4). (C) The mechanism of SARS-CoV-2 neutralization by MATCH-4.
ing affinity. With the inability to form multivalent binding, MATCH-1 (or free RBD aptamer) will easily dissociate from the RBD, so it is difficult to bind to three RBDs within one spike trimer at the same time. In contrast, with their well-matched valence and topological structures, MATCH-3 and MATCH-4 exhibit significantly improved binding affinities probably due to the active prompted-aptamer−RBD binding events in the presence of an aptamer bound to an RBD monomer, making it more likely that all three RBDs are bound by three aptamers assembled on MATCH-3 or MATCH-4.

Molecular Docking and Molecular Dynamics Simulations. To further understand the mechanism of the valence and topological matching interaction, we performed molecular docking and molecular dynamics simulations (MDS) to study the detailed interactions of MATCH-n and spike trimer (Figures S3 and S4). Considering the computational complexity, the 3D structures of MATCH-1−4 were simplified as a 5.8 nm equilateral triangle with 1−3-aptamers at the vertices: 1-aptamer for MATCH-1, 2-aptamer for MATCH-2, 3-aptamer for MATCH-3 and MATCH-4. Consistent with the \( K_d \) values, the absolute values of the simulated binding free energy change for the \( n \)-aptamer and spike trimer increased with the increase of the number of assembled aptamers. Interestingly, the 3-aptamer and spike trimer complex displayed an approximately 4-fold (not 3-fold) negative binding free energy change (−271 kcal/mol) compared to that of the 1-aptamer and spike trimer complex (−67 kcal/mol). The enhancement of binding energy above the multiple of valence states indicates that the clustered aptamer−spike binding is not a simple sum of the several individual aptamer−spike bindings, but that there is a synergistic effect for each individual aptamer−spike binding. As shown in Figure 3, compared to 1-aptamer binding, 3-aptamer showed not only three binding events, but also that the number of bases involved and the absolute value of the overall binding energy increased (Figure 3). Based on the experimental and simulation results, MATCH-4 has the best binding affinity against the spike trimer, so MATCH-4 was chosen for antivirus infection.

MATCH-4 Prevention of SARS-CoV-2 Pseudovirus from Entering HEK-293T-ACE2 Cells. To validate the interaction between MATCH-4 and SARS-CoV-2, a pseudotyped lentivirus packaged with the spike trimer gene of SARS-CoV-2 was chosen as the SARS-CoV-2 pseudovirus. Cryo-electron microscopy (cryo-EM) imaging was carried out to directly show the interaction between MATCH-4 and the SARS-CoV-2 pseudovirus. As shown in Figure 4A, MATCH-4 looks like a nanoscale “hat” which wraps over the spike trimer of the pseudovirus. Thus, MATCH-4 both enables topologically programmed multivalent aptamer binding and also provides appropriate steric hindrance of the DNA framework to further block the spike trimer and ACE2 interaction.

Time-lapsed, live confocal imaging was further applied to demonstrate that MATCH-4 prevents SARS-CoV-2 pseudovirus from entering the host cells without treatment with unblocking agent. Bottom row: MATCH-4 preblock pseudovirus entering cells. Scale bar: 20 \( \mu m \). (C) MATCH-4 blocks ACE2-293T and spike-293T cell fusion. ACE2-293T were dyed with Hoechst and spike-293T were transfected with GFP. Scale bar: 200 \( \mu m \).

Figure 2. (A) Flow cytometry analysis of the binding of MATCH-n with the spike trimer of SARS-CoV-2. (B) Binding curves and dissociation constants of MATCH-n against the spike trimer of SARS-CoV-2.

Figure 3. Results of molecular dynamics simulations of (A) 1-aptamer, (B) 2-aptamer, and (C) 3-aptamer. Histogram plot showing the interaction energy between the 1-, 2-, and 3-aptamer nucleotide and the spike trimer. The blue nucleotides were predicted to bind to the region on the RBD that mediated ACE2 receptor engagement.

Figure 4. Characterization of the binding of SARS-CoV-2 pseudovirus with MATCH-4. (A) Cryo-EM imaging of MATCH-4 binding with pseudovirus. Scale bar: 20 nm. (B) Time-lapsed, live confocal imaging. Top row: pseudovirus accumulated to enter the host cells without treatment with unblocking agent. Bottom row: MATCH-4 preblock pseudovirus entering cells. Scale bar: 20 \( \mu m \). (C) MATCH-4 blocks ACE2-293T and spike-293T cell fusion. ACE2-293T were dyed with Hoechst and spike-293T were transfected with GFP. Scale bar: 200 \( \mu m \).
Pseudovirus either pretreated with MATCH-4 or untreated was introduced to prestained ACE2-expressing HEK-293T cells. In the untreated situation, the accumulated pseudovirus bonded with and entered the host cells over time. In the cells pretreated with MATCH-4, pseudovirus accumulation was drastically reduced, and, even if some pseudoviruses reached the host cell, no obvious virus signal was observed from the cells over more than 1.5 h. Overall, these results established that MATCH-4 can bind to SARS-CoV-2 pseudovirus and prevent SARS-CoV-2 pseudovirus from infecting host cells.

**MATCH-4 Inhibition of 293T-ACE2 and 293T-Spike Cell–Cell Fusion.** In addition to the interaction of spike trimer and ACE2, the conformational transition of trimeric spike from a closed state to an open state is another crucial step associated with infection. Since MATCH-4 provides three spatially matched aptamers that can occupy three RBDs of the spike trimer, and the DNA framework will further restrict the swing motions of the spike trimer, MATCH-4 may inhibit SARS-CoV-2 fusion with the target cells. To investigate this, we used a SARS-CoV-2 spike-mediated cell–cell fusion assay, which is widely used to study virus and targeted cell fusion. 293T cells that can stably express SARS-CoV-2 spike protein (293T-S) and GFP were used as the effector cells, while 293T cells that express ACE2 (293T-ACE2) were chosen as the target cells. After 293T-S and 293T-ACE2 cells were cocultured for 24 h with or without MATCH-4 treatment, the number of 293T-S cells fused with 293T-ACE2 cells was counted. Compared to the group without MATCH-4 treatment, the fusion between 293T-S and 293T-ACE2 cells was significantly blocked by MATCH-4 (Figure 4C). In detail, the groups of untreated and phosphate-buffered saline (PBS)-treated 293T-S cells could fuse with 293T-ACE2 cells to form large syncytia, which could be easily observed by merging fluorescent images, while a smaller and less syncytium was shown when 293T-S were cocultured with 293T-ACE2 cells in the presence of MATCH-4. This result indicates that MATCH-4 inhibits 293T-ACE2 and 293T-S cell–cell fusion, leading to a reduction of spike trimer–ACE2 complex formation and the subsequent conformational transition of the spike trimer.

**Pseudotyped SARS-CoV-2 Neutralization.** We then evaluated the inhibition ability of MATCH-4 by the pseudovirus neutralization assay. The SARS-CoV-2 pseudovirus RNA genome contains luciferase and the GFP gene. Thus, virus-mediated infection can be determined via intracellular GFP or bioluminescence intensity. Briefly, the SARS-CoV-2 pseudovirus was incubated with different inhibitors, and the percentage of remaining infected cells was evaluated by GFP imaging.

The average inhibition efficiencies of MATCH-3 and MATCH-4 were ~82% and ~88%, respectively, both similar to a commercial neutralization antibody (87%, Research Resource Identifiers Number: AB_2857935) at the same dose (Figure 5A,B). In correlation with the IC₅₀ results, MATCH-1 and MATCH-2 provided a relatively lower inhibitory effect, with an inhibition efficiency of ~74.3% and ~79.1%, respectively. It is noteworthy that the monomer aptamer exhibits much poorer inhibition (~79.1%) than MATCH-1, even though they each contain one aptamer. Similarly, the inhibition efficiency of MATCH-2 is obviously better than that of the previous circularly bivalent aptamer at the same concentration against SARS-CoV-2 pseudovirus (Figure 5A), even though they have the same valence state.

**Mutated Pseudotyped SARS-CoV-2 Neutralization.** Recently, several new SARS-CoV-2 mutated variants have been rapidly spreading around the world, showing higher transmissibility and less susceptibility to some existing treatments or vaccines. In particular, the SARS-CoV-2 B.1.1.529 variant (Omicron) contains 15 mutation on the RBD and can escape the majority of existing neutralizing antibodies against SARS-CoV-2. Therefore, there is an urgent need to develop versatile strategies that can respond to different mutant strains.

With improved binding affinity and a dual blocking mechanism, MATCH-4 is predicted to have a higher mutation-resistant escape ability compared to the monovalent aptamer. As hypothesized, MATCH-4 retains >85% neutralization efficiency against several current prevalent mutant strains, including D614G, K417N: E484 K: NS01Y, L452R, E484Q; P681R, and Omicron mutant pseudovirus (Figure 6A–H). For example, 91% of the multisite mutant pseudovirus (L452R: E484Q; P681R) that mimics the dominant circulating Delta strain was blocked by MATCH-4 from infecting host cells, displaying much higher potency than the monomer aptamer and the neutralizing antibody (Figure 6G). For another current epidemic strain, the Omicron variant with 15 mutations on the RBD, MATCH-4 achieved nearly 100% neutralization (Figure 6H). Compared to other mutant strains, a 20 times higher...
treated with MATCH-4. It is noteworthy that most of the viral fluorescent signals with MATCH-4 treatment were on the infected cell membrane, and the intensity and area of viral signal in the cytoplasm were far less than those in the infected cells without MATCH-4 treatment, indicating that, although MATCH-4 could not completely prevent the authentic SARS-CoV-2 infection, it could still reduce the infection degree of the viruses.

To further demonstrate the potential of MATCH-4 for the treatment of SARS-CoV-2, the stability and biocompatibility of MATCH-4 were investigated. MATCH-4 is stable after storage at 4 °C for 15 days and demonstrated a decreased rate of degradation (Figure S6). When testing the safety of MATCH-4, the viability of cells was found unaffected by MATCH-4. More importantly, MATCH-4 did not elicit obvious cell cytotoxicity (Figure S7) nor immune response in vivo (Figures S8 and S9). Furthermore, MATCH-4 did not bind to white blood cells (Figure S10), suggesting no antibody-dependent enhancement mediated by the Fc fragment. Taken together, the potent inhibition efficiency of MATCH-4 against SARS-CoV-2 through synergetic spatially matched topological multivalent binding and steric hindrance exhibits excellent stability, biosafety, and immunogenicity, suggesting that MATCH-4 offers great potential for therapeutics of COVID-19 and other current or emerging coronaviruses.

CONCLUSIONS

In conclusion, considering the structural characteristics of the spike trimer and the infection mechanism of SARS-CoV-2, we have designed a DNA framework-guided spatially patterned multivalent neutralizing aptamer targeting SARS-CoV-2 neutralization. First, the well-matched topological structure and precise control of the neutralization aptamer organization contribute to significant improvement in binding affinity and neutralization activity. The neutralization effect of MATCH-2 against SARS-CoV-2 pseudovirus is better than that of the circularly bivalent aptamer at the same DNA concentration, even when each of them contains two aptamers. Moreover, the spatial- and valence-matched neutralizing aptamer assembly of MATCH-4 avoids the large amounts of waste disordered aptamers of the spherical neutralizing aptamer. Second, attachment of the DNA framework to the RBD—ACE2 binding interface further inhibits SARS-CoV-2 infection because of the steric hindrance from the ~5 nm DNA structure. As a result, compared to the monomer aptamer, the double blocking mechanism of spatially patterned multivalent binding ensures effective neutralizing activity of MATCH-4 against wild-type SARS-CoV-2 and its mutant strains, particularly the Omicron variant, which is evades neutralizing antibodies in the current pandemic. In addition, as binders specific to other targets can be evolved and assembled, the present design has the potential to inhibit wide-range and emerging pathogens.

One current limitation is that the structural information on the interaction between MATCH-4 and spike remains unknown. Rapid methodology development in structural characterization would promote the acquisition of such information, so as to further guide the MATCH-4 design to achieve a better neutralization effect. In addition, future work will be attempted to recruit biological systems to produce ssDNA massively and cost effectively and further evaluate the antiviral efficiency in vivo via intranasal/pulmonary delivery or...
injection, to advance the clinical application of MATCH-4 for COVID-19 treatment.

METHODS/EXPERIMENTAL

Materials. All DNA probes with HPLC purification were prepared by Sango Biotech (China). Ni beads for His-tagged RBD conjugation and Nap-5 Sephadex G-25 DNA grade columns were purchased from GE Healthcare (USA). His-tagged-Spike S1+S2 ECD recombinant of SARS-CoV-2 (40589-V08B1), His-tagged-RBD of SARS-CoV Spike/RBD protein (40150-V08B2), His-tagged-Spike/RBD of MERS-CoV protein (40071-V08B1), His-tagged-HIV-1 (40402-V08H1), His-tagged-human coronavirus HKU1 (40602-V08H1), His-tagged-SARS-CoV-2 Spike S1+S2 ECD protein (S614G) recombinant protein (40589-V08B4), and His-tagged-SARS-CoV-2 (2019-nCoV) Spike RBD (N501Y) recombinant protein were purchased from Sino Biological Inc. (China). AuNPs (5 nm) were purchased from Sigma Aldrich. Hoechst 33342 and Countess cell counting chamber slides were purchased from Thermo Scientific. Cell membrane dyes DiI and DiD were purchased from Mesgen and Beyotime. n-Luciferin potassium salt was from Yeasen. The carbon-coated 400-mesh copper grid was purchased from Electron Microscopy China. Glass-bottom plates were purchased from Fabio Biotechnology Co., Ltd. (Suzhou, China); the pseudovirus was discarded and fresh DMEM (10% FBS, 1% PS) was added. Then 48 h later, the culture medium was added to monolayer 293T-ACE2. After 6 h, the culture medium was removed and then 48 h later, the control group was incubated with pseudovirus in DMEM (10% FBS, 1% PS) at 37°C for 1 h. Then the mixtures were added into monolayer 293T-ACE2. After 6 h, the culture medium was discarded and fresh DMEM (10% FBS, 1% PS) was added. Then 48 h after the beginning of infection, images were observed by fluorescence microscope. For the IC₅₀ experiment, 48 h postinfection, n-luciferin potassium salt was added to 293T-ACE2, and the luciferase activity was measured by a multimode plate reader (SpectraMax iDS). The luciferase activity of the experimental group was divided by the luciferase activity of the control group and the IC₅₀ value was calculated by Prism software.

For the mutated pseudotyped SARS-CoV-2 inhibition assay, 20 nM MATCH-4 was incubated with SARS-CoV-2 Spike S1+S2 ECD recombinant protein (N501Y) and the PMK-4 pseudovirus; for Omicron inhibition assay, 400 nM MATCH-4 was incubated with Omicron pseudovirus. The concentration of the corresponding antibody was the same as that of MATCH-4. The monomer aptamer concentration is four times that of MATCH-4.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.2c06803.

Yield analysis of TDF, MATCH-n assembly; TEM images of the MATCH-n assembled with quantitative AuNPs; the RMSD for each assembly; docking complexes of 1–3-aptamer binding with SARS-CoV-2 RBD; time-lapse, live confocal imaging; stability analysis of MATCH-4; H&E staining images; serum levels of IFN-α in mice; MATCH-n binding to white blood cells; and sequences of oligonucleotides (PDF).
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S.W. and S.L. contributed equally to this work.

Notes
The authors declare no competing financial interest.

This manuscript has been previously submitted to a preprint server as Shuang Wan; Siwen Liu; Sun Miao; Jialu Zhang; Xinyu Wei; Ting Song; Yuhao Li; Xinyang Liu; Honglin Chen; Chaoyong Yang; Yanling Song. Spatial and Valence Matched Support. CAMBRIDGE: Cambridge Open Engage. https://chemrxiv.org/server as Shuang Wan; Siwen Liu; Sun Miao; Jialu Zhang; Xinyu Wei; Ting Song; Yuhao Li; Xinyang Liu; Honglin Chen; Chaoyong Yang; Yanling Song. Spatial and Valence Matched Support. CAMBRIDGE: Cambridge Open Engage. https://chemrxiv.org/server

ACKNOWLEDGMENTS
We thank the National Natural Science Foundation of China (Grants 22024209, 21735004, 21874089, 21775128) and the Program for Changjiang Scholars and Innovative Research Team in University (Grant IRT13036) for their financial support.

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