

SINGLE-DOMAIN ANTIBODY FOR BINDING THE CONSERVED EPITOPE IN THE SARS-COV-2 SPIKE PROTEIN RECEPTOR-BINDING DOMAIN

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Several COVID-19 vaccines have been developed in the last three years using various techniques. Multiple virus-neutralizing antibodies against SARS-CoV-2 have been also obtained to combat the pandemic. However, the use of these medications for prevention and potential treatment faces significant challenges due to the emergence of new mutant virus variants, both more contagious and escaping neutralization by the immune system, that is why it is necessary to continuously renew the vaccines and develop new therapeutic antibodies. The study was aimed to use the technology of generating single-domain antibodies (nanobodies) to target the surface spike (S) protein RBD conserved epitope of the broad spectrum of SARS-CoV-2 variants. Recombinant proteins that corresponded to RBDs of three important SARS-CoV-2 strains and the full-length S protein (Wuhan) were used as antigens for immunization of a camel in order to induce production of appropriate antibodies and/or as immobilized proteins for further cross selection of the nanobody clones with pre-set specificity by the phage display. A nanobody capable of effectively recognizing the conservative region in the S protein RBDs of the broad spectrum of pandemic SARS-CoV-2 variants, including Omicron, was selected from the generated immune library. Along with conventional use in immunoassays and diagnosis, the generated nanobody can be potentially used as a module for target-specific binding used to trap coronavirus in human upper airways during the development of novel combination antiviral drugs.

Keywords: SARS-CoV-2, conserved epitope, single-domain antibody, nanobody, virus retention

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ОДНОДОМЕННОЕ АНТИТЕЛО ДЛЯ СВЯЗЫВАНИЯ КОНСЕРВАТИВНОГО ЭПИТОПА РЕЦЕПТОР-СВЯЗЫВАЮЩЕГО ДОМЕНА БЕЛКА SPIKE КОРОНАВИРУСА SARS-COV-2

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В последние три года с помощью разных технологий были разработаны несколько вакцин против COVID-19, а также получено большое число вирус-нейтрализующих антител к коронавирусу SARS-CoV-2 с целью борьбы с пандемией. Однако применение этих препаратов для профилактики и потенциального лечения сталкивается с существенными проблемами из-за появления новых мутантных вариантов вируса, как более контагиозных, так и ускользающих от иммунной нейтрализации, что постоянно требует обновления вакцин и разработки новых терапевтических антител. Целью данного исследования было использовать технологию создания однодоменных антител (нанотел) для таргетирования наиболее консервативных эпитопов рецептор-связывающего домена (RBD) поверхностного S-белка (шипа, Spike) широкого спектра вариантов SARS-CoV-2. Рекombинантные белки, соответствующие RBD трех актуальных штаммов SARS-CoV-2, а также полноразмерному S-белку (Wuhan), были использованы в качестве антигенов для иммунизации верблюда с целью индукции образования соответствующих антител и/или в качестве иммобилизуемых белков для последующих перекрестных процедур селекции клонов нанотел с заданной специфичностью методом фагового дисплея. Из генерированной иммунной библиотеки было отобрано нанотело, обладающее свойством эффективно узнавать консервативный участок RBD S-белка широкого спектра вариантов пандемического коронавируса SARS-CoV-2, включая омикрон. Помимо традиционного использования в иммуноанализе и диагностике, полученное нанотело потенциально может быть использовано в качестве модуля мишень-специфичного связывания для задержки коронавируса в верхних дыхательных путях человека при разработке новых комбинированных противовирусных препаратов.

Ключевые слова: SARS-CoV-2, консервативный эпитоп, однодоменное антитело, нанотело, задержка вируса

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Most virus neutralizing antibodies against important targets, such as SARS-CoV-2, bind to protein epitopes on the surface of viral particles and prevent the interaction of virus with the cellular receptor that is essential for viral entry. As for SARS-CoV-2, the spike (S) protein (1300 amino acids) displayed on the surface is the main target for neutralizing antibodies. S protein forms a homotrimer on the virion surface. During virus assembly this protein is broken down into N-terminal (S1) region and C-terminal region (S2) that is directly involved in fusion with the host cell membrane. A receptor-binding domain (RBD, 319–541 amino acids or at least 333–527 amino acids) has been identified in the S1 region. In the S protein open conformation, RBD interacts with the angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 entry receptor displayed on the host cell surface. The latter triggers S protein rearrangement that results in membrane fusion and viral entry. RBD consists of two core subdomains with the central β -sheet and one external subdomain or the receptor-binding motif (RBM, amino acids 438–506) that is linked to two adjacent β -strands of the core; RBM becomes the region of S protein in its receptor-binding open conformation that is most distant from the viral surface. RBD (and particularly RBM) is the main antigenic region of S protein and the main target for the virus neutralizing antibodies [1–3].

From the beginning of the pandemic the currently circulating SARS-CoV-2 strains have acquired mutations relative to the original Wuhan (WA1) strain. In particular, the B.1.617.2 variant (Delta) [4] and the related B.1.617.1 variant (Kappa) first identified in India carry the RBD L452R-T478K and L452R-E484Q substitutions, respectively, that are probably responsible for enhanced infectiousness [4]. The B.1.1.529 variant (Omicron) identified in South Africa comprises the extension of RBD with 15 substitutions (such as G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A Q493K, G496S, Q498R, N501Y, Y505H) [5]. RBD mutations found in these variants increase the risk of reducing the effectiveness of modern vaccines and therapeutic human antibodies. These mutations can also contribute to virus evolution and selection of new variants capable of escaping neutralization by human immune system. Thus, generating the broad-spectrum virus neutralizing antibodies against various SARS-CoV-2 strains (existing and future) is extremely challenging [6–8].

The use of antibody-based inhalation medications is a very promising method for targeted interventions and combating respiratory infections at the point of entry into the human body, i.e. in the upper airways [9]. Inhalation is a promising non-invasive strategy for delivery of antibodies used for treatment of respiratory diseases, since this route ensures higher antibody concentrations in the respiratory tract, thereby overcoming the constraints and uncertainty related to the drug levels in the right area after systemic antibody delivery through the bloodstream. The nasal route of drug delivery is one of the well-characterized administration routes. For a number of drugs, the nasal sprays were considered successful and approved for widespread use. Respiratory viruses that infect humans enter the body through the respiratory tract in aerosols produced by the other infected individuals' cough or sneezing. Large aerosol particles are usually retained in the turbinates and sinuses, where these particles may cause upper respiratory infections. The smaller particles can travel to the lower respiratory tract and cause more dangerous infections affecting the alveolar region. The majority of viruses that infect the upper respiratory tract cause acute infections and show seasonality (for example, respiratory syncytial virus (RSV), rhinovirus, parainfluenza and influenza A viruses, adenovirus, human metapneumovirus, human bocavirus, and coronavirus). Mucosal epithelial cells represent

the portal of entry for most respiratory viral infections. The virus would not be able to initiate the infection, if its attachment to the cell is blocked at the point of entry. It has been shown that the influenza virus particles can be trapped in the human respiratory mucus, regardless of haemagglutinin binding sialic acids on mucins [10]. Perhaps the trapping observed is due to the presence of antibodies binding both influenza virus and components of the mucus gel. The link between antibodies and the mucus gel is probably ensured through the antibody Fc fragment multiple low affinity interactions with mucin. It is important to note that adhesive interactions between the antibodies that bind pathogens and the components of respiratory mucus may provide a universal strategy for combating pathogens in the airways. The topical delivery of antibodies that bind the causative agents of respiratory infections can potentially decrease the risk of infection and reduce viral load in the respiratory epithelium.

The format of single-domain antibodies is one of the most promising formats of monoclonal antibodies that gains more attention in recent years, including in the context of developing new means of combating infections.

The recombinant derivatives of the single-domain antigen-binding fragments (VHH) of the specific HCAb antibodies (heavy-chain only antibodies) comprising the truncated heavy chain dimer and no light chains, that are normally found in blood of the Camelidae family members and some species of cartilaginous fishes in addition to conventional immunoglobulin types, are referred to as single-domain antibodies (nanobodies) [11–12]. The main features of nanobodies are as follows: small size (12–15 kDa, 4 × 2.5 nm); high solubility, stability, specificity, and affinity; thermal and chemical resistance; easy realization of various modifications by genetic engineering methods; possibility of using the extremely effective phage display method for selection of optimal nanobody variants. Nanobodies are capable of forming unique paratopes and recognizing unique native antigen epitopes that are unusual for conventional antibodies (mostly conformational epitopes, small grooves, active sites of enzymes). This may result in the extraordinary high specificity of the specified target recognition *in vivo*. The framework regions of the camelid nanobodies (VHH) have high homology (significantly higher compared to the mouse VH) with the framework regions of the variable VH domains of human immunoglobulins (IgG3 subclass). Nanobody production in bacteria or any other expression systems is very cost-effective; nanobodies can be used as building blocks for multi-domain constructs [13–14].

The promising nanobody-based antiviral therapeutic drugs have already been created. For example, several nanobodies have been obtained that specifically bind to the most conserved fragments of haemagglutinin of the influenza viruses of various subtypes [15]. The multi-domain constructs containing four different nanobodies have been inserted in the adeno-associated virus vector.

The mouse model has shown that the expressed multi-domain antibodies targeted at several conserved epitopes at once very effectively prevent infection with influenza A and B viruses. The same strategy can be used for prevention of infection with other viruses/pathogens that show high variability. It is important to note that no such medication has not yet been obtained using conventional monoclonal antibodies.

A number of recently published papers report generating nanobodies against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein in order to block its interaction with ACE2 and thereby neutralize the virus [16–17]. This was accomplished for many SARS-CoV-2 variants, except for the most heavily mutated Omicron strain.

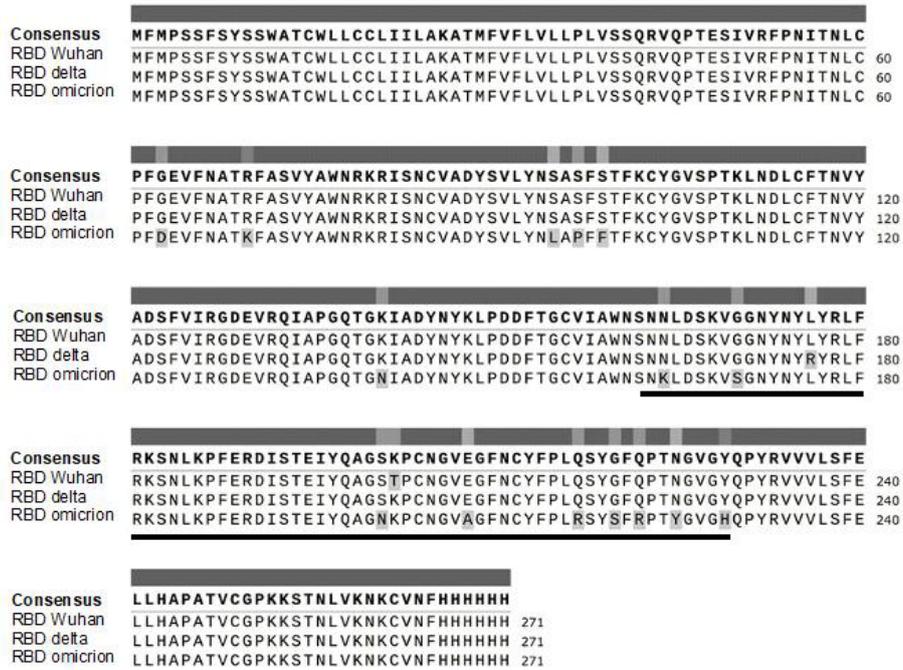


Fig. 1. Scheme of the aligned amino acid sequences of RBDs of three SARS-CoV-2 S protein mutant variants (original RBD Wuhan, RBD Delta, and the most heavily mutated RBD Omicron). Mutations of amino acid residues are highlighted in gray. The bold line represents position of the receptor-binding motif (RBM) that interacts directly with the ACE2 receptor

There is a report of generating the nanobody (Nb6) that binds and blocks S protein in a fully inactive conformation, thus preventing the virus binding to ACE2 [18]. Affinity maturation *in vitro* and trimerization of the high-affinity nanobody derivative have made it possible to obtain a drug against the SARS-CoV-2 infection that shows picomolar neutralization activity. This drug remained stable and retained its function after aerosolization, lyophilization, and heat treatment. The authors assume that

the aerosolized delivery of this potent neutralizer directly to the respiratory epithelium is possible.

The study was aimed to use the antibody-producing technology for selection of nanobody against the S protein RBD conserved epitope of the broad spectrum of SARS-CoV-2 variants, including Omicron. Such a nanobody is considered as a potential virus-binding module of the future aerosol combination virus-trapping drug that would contain an anchor

Table 1. Oligonucleotides (primers) for point mutagenesis

Primer	Nucleotide sequence 5'-3'
<i>For RBD Delta</i>	
RBD delta for 63	atctgaagcccttcgagcgggacatctccaccgagatctatcaggccggcagcaaaccttg
RBD flank rev 63	tctgctagctcgagtcgagc
Vector for 59	atcttaagtcgagctcgagc
Vector rev 59	gtcggccacgcaattgc
RBD new rev 55	gtggagatgtcccgctcgaagggctcagattggacttccggaacagccggtatctgtaattg
RBD new flank for 53	gtggagatgtcccgctcgaagggctcagattggacttccggaacagccggtatctgtaattg
<i>For RBD Omicron</i>	
LIC ins for II 65	ccccttcGACgaggtgttcaatgccaccAAGttcgctctg
LIC ins omi I for 61	ctgtacaacCTCgccCCGttCTTtacctcaagtgtcaccggctgtcccc
LIC ins omi I for 63	gggctgATGccccacgccGTAtgtgggCCGaaaACTgtaggaTCGcagtGGGAAG
LIC ins omi II rev 65	ttgaagccTGCcacgccgttacaaggAAAGTTgccggcctgatag
LIC vec omi I for 58	CaTACggcgtggcCatcagccc
LIC vec omi I rev 59	AAAgaacCGggcGAGgtttacagcagcggagtagtcg
LIC vec omi II for 63	ttgtaacggcgtgGCAggcttcaactgc
LIC vec omi II rev 65	attgaacacctcGTCgaaggggcacagattg
SDM II omi for 55	gtggcaactacaattacAGAtaccggctgttcc
SDM II omi rev 56	TgactttgagtcagTTTgttctg
SDM omi for 54	atatcggcactacaactac
SDM omi for 55	tgctgtctgtccagg
CAG seq for rbd	tcctgggcaactgctgg
CAG seq rev rbd	agatgctcaaggggc

module for binding the major components of the human upper respiratory tract secretions.

METHODS

Production of recombinant proteins corresponding to the S protein receptor-binding domains of three important SARS-CoV-2 strains and the full-length S protein (Wuhan)

The SARS-CoV-2 strain (Wuhan) RBD was produced using the NR-52309 plasmid provided by Krammer et al. [19] (BEI Resources web-site, NIAID, NIH) in the HEK293T eukaryotic cells in accordance with the protocol published on the web-site and as previously reported [20]. The designated vector for the SARS-CoV-2 RBD, Wuhan-Hu-1 (GenBank: MN908947), was generated by fusing the S protein N-terminal signal sequence with RBD (amino acids 319–541) and the C-terminal hexahistidine tag. The target RBD-encoding plasmid was introduced into the HEK293T cells by calcium phosphate transfection. Three days later the RBD protein-containing supernatant was collected and centrifuged at 4 °C and 1500 g for 10 min. The purified supernatant was mixed with 5 mL of Ni-NTA-agarose (Qiagen; USA) equilibrated in phosphate-buffered saline and subsequently incubated with continuous mixing on the orbital shaker (Biosan; Latvia) for an hour at room temperature. Suspension was applied to the column and subsequently washed with the wash buffer (57 mmol NaH₂PO₄·H₂O, 135 mmol NaCl, 20 mmol Imidazol), then RBD was flushed with elution buffer (57 mmol NaH₂PO₄·H₂O, 135 mmol NaCl, 235 mmol Imidazol). The protein obtained was dialyzed against phosphate-buffered saline using a 5 kDa dialysis membrane (Merck; USA). The purified protein was analyzed by polyacrylamide gel electrophoresis. The protein concentration was determined by normalization with the known concentrations of bovine serum albumin (BSA) and by the Pierce BCA protein assay (Thermo Fisher; USA). Absorbance was measured using the CLARIOstar plate reader (BMG Labtech; USA).

To obtain RBDs of the SARS-CoV-2 Delta and Omicron strains, we introduced appropriate nucleotide substitutions (highlighted in Fig. 1) in the original sequence encoding RBD comprised by the above mentioned plasmid. Substitutions were introduced using the specially synthesized oligonucleotides by ligation-free cloning through synthesis of overlapping intermediate PCR products using the T100 Thermal cycler (Bio-Rad; USA). After amplification of appropriate fragments using the PhusionTM high-fidelity DNA polymerase (Thermo Fisher; USA), the amplified fragments were subjected to 1% agarose gel electrophoresis, and the fragments cut were isolated using the Cleanup Standart kit (Evrogen; Russia). The amplified vector and insert were incubated with T4 polymerase at 37 °C for 3 min, then T4 polymerase was inactivated at 75 °C for 15 min followed by incubation on ice for 1 min on ice for 1 min. After that the resulting mixture was used for transformation of the TOP10 competent cells. RBD was expressed and purified as previously described [20].

Oligonucleotides used for mutagenesis are provided in Table 1 (the coordinates correspond to that provided in Fig. 1). As for RBD Delta variant, mutations L176R, T202K were reproduced. To synthesize the sequence encoding the SARS-CoV2 Omicron strain RBD, the nucleotide substitutions that corresponded to mutations G63D, R70K, S95L, S97P, S99F, K141N, N164K, G170S, S201N, T202K, E208A, Q217R, G220S, Q222R, N225Y, Y229H, were introduced in the original plasmid encoding the SARS-CoV-2 strain RBD.

The sequences obtained by cloning were tested by Sanger sequencing using the RBD new flank for 53 or CAG seq oligonucleotides (Table 1).

The full-length SARS-CoV2 S protein was expressed and purified in accordance with the earlier reported protocol [19, 20] as described above, with the difference that the spike protein was expressed instead of RBD (using the pSFHT pCAGGS-wt Spike-Trb-T4-HT plasmid DNA provided by Krammer). Production and purification were performed in the same manner as described for RBD [20].

Immunization and acquisition of the library of cDNA sequences encoding nanobodies

The camel was kept in the spacious enclosure with regular paddock and feeding in the Center for Collective use “Live Collection of Wild Mammals” at the Scientific and Experimental Base “Chernogolovka”, Severtsov Institute of Problems of Ecology and Evolution of the Russian Academy of Sciences. Animal work was carried out in accordance with the National Standard of the Russian Federation GOST R 53434–2009. The Bactrian camel (*Camelus bactrianus*) was sequentially immunized with five doses (four weeks after the first injection, then each subsequent injection was performed within 10–14 days) by subcutaneous injection of antigenic material mixed with equal amount of complete (first injection) or incomplete (other injections) Freund's adjuvant. A total of 0.5 mg of the mixture of recombinant proteins corresponding to RBD Wuhan and RBD Delta were used as antigenic material for immunization. Blood (150 mL) was collected five days after the final injection. To prevent blood clotting, 50 mL of the standard phosphate-buffered saline (PBS) containing heparin (100 U/ml) and EDTA (3 mmol) were added. Blood was two-fold diluted with PBS containing 1 mmol EDTA; 35 mL of diluted blood were layered on the 15 mL step of specialized medium (Histopaque-1077; Sigma-Aldrich, USA) with the density of 1.077 g/mL; centrifugation at 800 g was performed for 20 min. Mononuclear cells (lymphocytes and monocytes) were collected from the plasma/Histopaque interphase zone and subsequently washed with PBS containing 1 mmol EDTA. Total RNA was extracted from B cells using TRIzol (Thermo Fisher Scientific; USA). Then poly(A)-containing RNA was purified from total RNA using the Oligo(dT)-cellulose column. RNA concentration was defined with BioPhotometer (Eppendorf; Germany), and the quality of extracted RNA was verified by 1.5% agarose-formaldehyde gel electrophoresis. The reverse transcription reaction was carried out using the Maxima reverse transcriptase (Thermo Fisher Scientific; USA) and the Oligo(dT)18 primer. The reverse transcription products were used as a matrix for two-step polymerase chain reaction, and amplification products were cloned into a phagemid vector with the NcoI(PstI) and NotI sites as previously described [21]. The pHEN4 expression vector [22] kindly provided by Professor S. Muyldermans (Vrije Universiteit Brussel, Belgium) was used for cloning.

Selection of the nanobody clones that bind to RBD; formatting, production, and analysis of anti-RBD nanobodies

The next phage display-based selection procedures involving the use of the M13KO7 bacteriophage (New England Biolabs; USA) as a helper phage were mainly conducted in the same way as the previously described procedures [21].

The Excella E24 and E25 shaker incubators (NewBrunswick Scientific; USA) were used to grow the bacterial culture. The 5810R and 5415R refrigerated centrifuges (Eppendorf;

Germany) were used for centrifugation. The recombinant proteins obtained by the above methods were immobilized in the wells of the Maxisorp immuno plate (Nunc; Denmark). The wells were blocked with 1% BSA (bovine serum albumin) in 1× PBS or with the casein blocking buffer (Sigma-Aldrich; USA). Both alternate antigens and blocking proteins were used for sequential selections. The selected clones of the nanobody-encoding sequences were grouped based on the identity of the HMR-fingerprint-like images and the activity of the expressed nanobodies (with the C-terminal HA tag) in periplasmic extracts [21]. The promising clones were re-cloned adding to the C-terminus of the nanobody-encoding sequences additional sequences of the long hinge region (as a linear flexible linker), HA tag and His tag for detection and effective purification of nanobodies, as previously described [23]. All the expression constructs comprised the pelB leader sequence for periplasmic nanobody expression. This made it possible to isolate the nanobody by the osmotic shock method without disrupting bacterial cells. Nanobodies were expressed in the *E. coli* cells (XL1 strain). Protein expression in the exponentially growing cells was induced by adding 0.2-1 mmol IPTG (isopropyl β -D-1-thiogalactopyranoside). The cells were incubated with vigorous agitation for 5 h at 30 °C and overnight at 28 °C. The recombinant proteins were extracted from the periplasmic extract by affinity chromatography with Ni-NTA-Agarose (Qiagen; USA) in accordance with the manufacturer's instructions. The periplasmic extract containing a nanobody with C-terminal HA tag or an affinity purified adapted nanobody were used to assess specificity and efficiency of the nanobody binding to the antigen preparation immobilized in the immune plate well by conventional enzyme-linked immunoassay (ELISA). The horseradish peroxidase-conjugated anti-HA tag monoclonal antibody (H6533, Sigma-Aldrich; USA) was used as a secondary antibody against the HA tag. The horseradish peroxidase activity was defined with the use of 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific; USA) as a chromogenic substrate. Absorbance was measured at 450 nm with the Multiscan EX photometric microplate absorbance reader (Thermo Labsystems; USA) after adding equal volume of sulfuric acid (2 mol). The control wells contained no antigen, however, these were blocked and processed in parallel with the experimental wells (antigen-containing). Commercially available preparations were used for competitive nanobody assay: XR19 neutralizing mouse monoclonal nanobody (Xema; Russia) that is currently under approval and is manufactured as an experimental batch, and peroxidase conjugated rabbit anti-mouse immunoglobuline antibody (IMTEK; Russia). A measurement was taken three times, and the ELISA results were presented as mean values and standard deviations that did not exceed 10%.

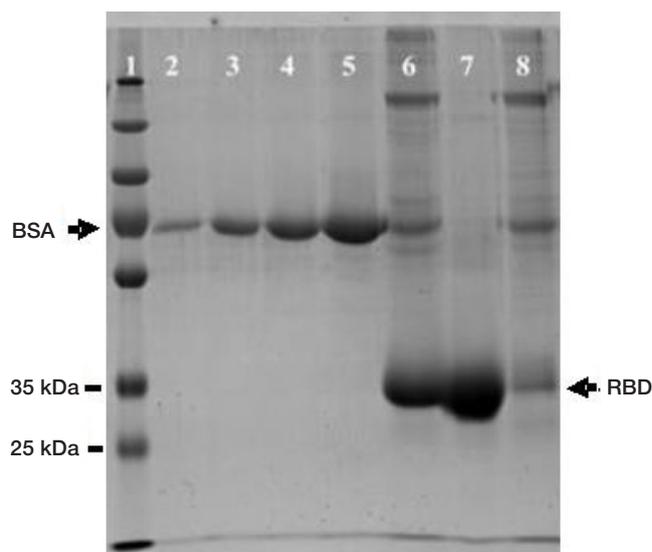


Fig. 2. SDS-polyacrylamide gel electrophoresis showing the produced (using the cloned coding sequence) and subsequently purified recombinant RBDs of three strains (6 — RBD Delta, 7 — RBD Wuhan, 8 — RBD Omicron). Left — lane of marker proteins (Thermo Scientific PageRuler Plus Prestained Protein Ladder, size 10–250 kDa). Different amounts of BSA marker protein have been applied to lanes 2–5 in order to quantify the protein produced (0.25, 0.5, 1.0, and 2.0 mg, respectively)

RESULTS

Initially we cloned and produced recombinant proteins corresponding to the S protein receptor-binding domains of three important SARS-CoV-2 strains (Fig. 2). Yield of produced proteins per 10^8 cells: RBD delta — 2200 ng, RBD Wuhan — 2446 ng, RBD omicron — 300 ng, membrane-associated full-length S-protein Wuhan (Fig. 3) — 75 ng.

Recombinant proteins that corresponded to RBD Delta and RBD Wuhan were produced rather effectively, however, significantly lower reproducible protein output was observed for RBD Omicron. This was probably due to the features of the RBD Omicron protein secondary structure that could be formed harder in this expression system. When we started this study, no information about the RBD Omicron was available, that is why the mixture of RBD Delta and RBD Wuhan proteins was used for immunization of the camel. RBD Omicron was later used at the selection stage.

The recombinant protein produced that corresponded to the full-length SARS-CoV-2 (Wuhan) S protein was used at the same stage of selection and validation ELISA (Fig. 3).

After the Bactrian camel immunization, the titer of IgG binding to RBD Delta and RBD Wuhan in the antiserum

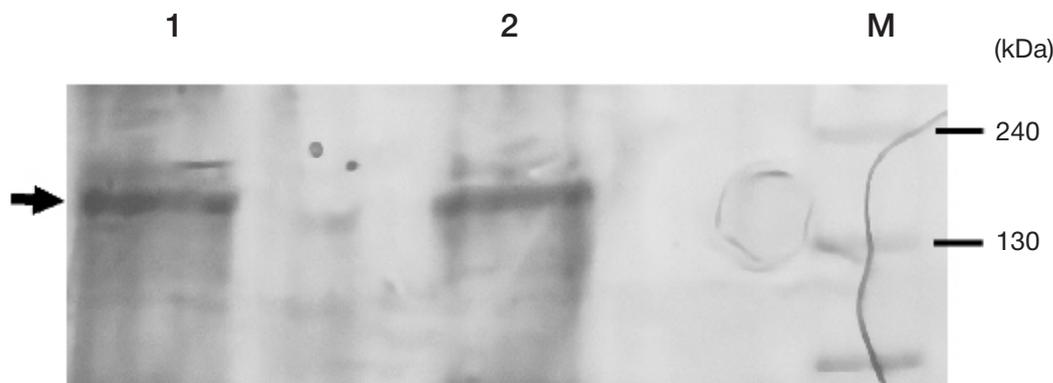


Fig. 3. SDS-polyacrylamide gel electrophoresis showing the produced SARS-CoV-2 (Wuhan) recombinant S (spike) protein. The protein was detected in the cytoplasmic fraction (1) and in the sediment fraction (2). The marker of the polypeptide molecular weight is applied on the right

significantly (about 40 times) increased compared to pre-immune serum. We cloned cDNA sequences encoding the entire repertoire of the single-domain antigen-recognizing sequences (VHH, single-domain antibodies, nanobodies) containing a homodimer of truncated (having no CH1 domain) heavy chains and no light chains in the pHEN4 phagemid expression vector based on mRNA from the immunized camel peripheral blood lymphocytes. The resulting library of the VHH-cDNA sequences was converted into the phage particle format using the M13KO7 helper phage and then used for cross selection with three variants of produced RBD. Selection was performed by sequentially using the immune plate wells showing high sorption capacity (Nunc Maxisorp) that contained recombinant proteins immobilized in PBS with a concentration of 10 $\mu\text{g}/100 \mu\text{L}$ (RBD Wuhan, RBD Delta, and RBD Omicron). The 1% BSA (Sigma-Aldrich; USA) in PBS or the casein blocking buffer (Sigma-Aldrich; USA) were used for blocking. Selection and subsequent amplification of the selected phage particles (containing the gene encoding the single-domain nanobody inside and the expressed single-domain nanobody as a component of the pIII phage surface protein) were usually performed three times in a row using different incubation sequences for all three variants of the immobilized antigens. The first two RBD variants were used for immunization, and it was easy to select nanobodies against these variants. However, it was predictably difficult to select a nanobody capable of effectively binding RBD Omicron as well. The sequences of selected nanobody clones were grouped based on the similarity of fingerprints obtained by electrophoretic separation of the products of hydrolysis of the amplified sequences of single-domain nanobodies using three restriction endonucleases (HinfI, MspI, RsaI) at once. To assess specific activity of the selected nanobodies that represented each group, microinduction of the nanobody synthesis in the bacterial periplasm was performed, and the periplasmic extract containing the generated nanobodies was obtained. Such periplasmic extracts with nanobodies comprising a C-terminal HA tag (antigenic determinant, the YPYDVPDYA fragment of 9 amino acids) were used when performing enzyme-linked immunoassay (ELISA) for detection of the most promising nanobody variants. The horseradish peroxidase-conjugated

anti-HA tag monoclonal antibody (H6533, Sigma-Aldrich; USA) was used as a secondary antibody against the HA tag. We managed to select nine different variants (groups) of the nanobody clones after selection and ELISA. Among those the most promising nanobody referred to as aRBDce1 (anti-RBD conserved epitope 1) that met all the major requirements we had initially set should be highlighted.

The selected cloned nanobody-encoding sequences were adapted for more effective production in the bacterial expression system and subsequent effective nanobody purification, as described earlier [23]. The nanobodies obtained comprise a long C-terminal linker sequence (28 amino acids of the long variant of the non-conventional camelid antibody hinge region) followed by two peptide fragments: HA tag allowing to detect the nanobody using the commercially available antibodies against this peptide and (His)₆ tag, the sequence of six histidine residues, that makes it possible to effectively purify proteins containing the tag by the metal-chelate affinity chromatography on the Ni²⁺-NTA agarose. The selected and adapted nanobodies were tested for functional activity. Initial testing was performed by ELISA in order to test the efficiency of the S protein RBD conserved epitope binding by the nanobodies obtained for three different mutant SARS-CoV-2 variants. Fig. 4 shows the ELISA results suggesting that the aRBDce1 nanobody (1 $\mu\text{g}/\text{mL}$) is highly effective: it binds to the recombinant S proteins, RBD Wuhan (W-RBD), RBD Delta (Δ -RBD), and RBD Omicron immobilized in the plate wells better than other nanobody variants selected alongside, however, it hardly binds to the control well. The wells with immobilized BSA (bovine serum albumin) were used as controls. The signal intensity reflects the nanobody binding efficiency. We can assume higher affinity of the aRBDce1 nanobody binding to RBD (in the low nanomolar range) compared to other earlier generated in one group and characterized nanobodies against other targets; aRBDce1 is so far the only nanobody we have selected that is capable of effectively binding all the RBD variants used, including RBD Omicron and the full-length S protein.

Fig. 5 shows the results of ELISA, during which the immobilized RBD (Wuhan) was first bound/blocked with the increasing concentrations of individual variants of the selected nanobodies (20 $\mu\text{g}/\text{mL}$), then the wells were washed, and a

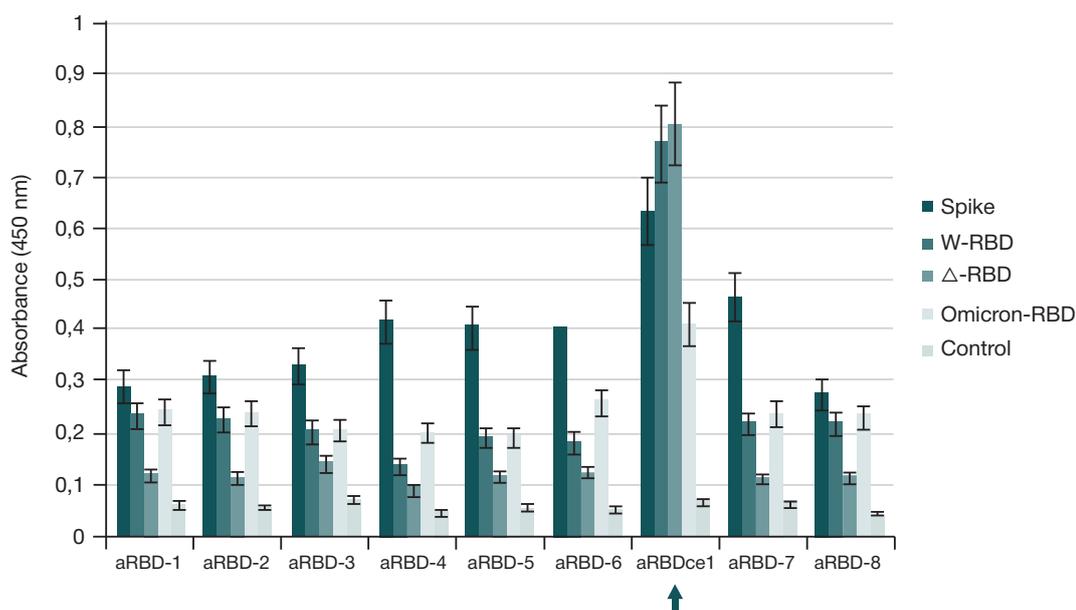


Fig. 4. Results of ELISA of nine selected nanobody variants (aRBD-1–aRBD-8 and aRBDce1) binding to immobilized recombinant proteins that correspond to (left-to-right) spike protein, RBD Wuhan (W-RBD), RBD Delta (Δ -RBD), and RBD Omicron, and to the control well with no antigen blocked with 1% BSA (as in all other wells). The absorbance values reflect the effectiveness of nanobody binding. Mean values for three independent experiments and standard deviations are provided

region (S2) that is directly involved in fusing with the host cell membrane. However, our unpublished data on the nanobodies against the similar region of the influenza virus haemagglutinin suggest that there could be some spatial constraints that prevent the nanobody from reaching such epitopes *in vivo*. The S protein RBD region comprises the epitopes that are most accessible for binding. Furthermore, it contains regions with the relatively low mutation rate [6–8]. We have chosen this RBD region as the initial target, because this region is potentially the most accessible for binding with the nanobody that is considered as a potential virus-binding module of the future aerosol combination virus-trapping medication that would also contain an anchor module for binding with the major components of human upper respiratory tract secretions. We assume that it is a good idea to have several binding modules against various epitopes to be less dependent on the new mutations. Moreover, in the future we consider the use of inactivated whole virus particle as the

set of antigens most close to native set for immunization and subsequent selection aimed at using the approach similar to that reported in this paper to generate the nanobodies against the conserved surface epitopes of the virus.

CONCLUSIONS

Thus, the paper reports the method to obtain nanobodies (aRBDce1) against the conserved epitope of the receptor-binding domain of the SARS-CoV-2 main surface protein, the spike protein, using the recombinant proteins that correspond to this domain in various most relevant mutant virus variants and the effective technique for generating single-domain antibodies. Along with conventional use in immunoassays and diagnosis, the generated nanobody can be potentially used as a module for target-specific binding of coronavirus during the development of novel combination antiviral drugs.

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