Journal of Pharmaceutical Sciences 112 (2023) 1664–1670

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Immunogenicity Evaluation of Thermostable Microparticles Entrapping Receptor Binding Domain of SARS-CoV-2 by Single Point Administration

Rahul Ahuja[a,b,*], Sudeepa Srichandan[a], Jairam Meena[a,c], Bichitra Kumar Biswal[d], Amulya K. Panda[a,1,*]

[a] Product Development Cell, National Institute of Immunology, New Delhi, 110067, India
[b] Infection and Immunology Laboratory, Translational Health Science & Technology Institute, Faridabad, Haryana, 120001, India
[c] Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University), Uttar Pradesh, 221005, India
[d] Structural and Functional Laboratory, National Institute of Immunology, New Delhi, 110067, India

ARTICLE INFO

Article history:
Received 12 July 2022
Revised 25 January 2023
Accepted 25 January 2023
Available online 2 February 2023

Keywords:
SARS-CoV-2
Receptor binding domain (RBD)
Polymer particle
Thermostable vaccine
Immunogenicity

ABSTRACT

Receptor binding domain (RBD) of SARS-CoV-2 is a prime vaccine target against which neutralizing antibody responses are directed. Purified RBD as a vaccine candidate warrants administration of multiple doses along with adjuvants and use of delivery systems to improve its immunogenicity. The present investigation examines the immunogenicity of RBD delivered by biodegradable polymer particles from single dose administration. Mice upon single point immunization of RBD entrapped microparticles generated improved antibody response. The polymer microparticles showed better temperature stability and could be stored at 37 degrees for one month without any considerable loss of immunogenicity. Further, immunization with microparticles could elicit memory antibody response upon challenge after four months of single dose administration. Thus, using microparticles entrapping RBD as a vaccine candidate confer improved immunogenicity, temperature stability and recall response. These thermostable microparticles seem to be a potentially cost-effective approach which can help in dose reduction, provide a wider access of vaccines and accelerate the end of global pandemic.

Introduction

The humankind is facing global pandemic caused by SARS-CoV-2 which has led to 189.74 million cases and 4.08 million mortality.[1] SARS-CoV-2 emerged in Wuhan, China, December 2019 and soon spread across the globe. SARS-CoV-2 was declared as Public Health Emergency of International Concern (PHEIC) by the WHO by end of January 2020.[2] As per recent estimates, SARS-CoV-2 claims nearly 5000 lives every day.[3-5] SARS-CoV-2 is an enveloped virus containing RNA genome. The viral spike protein forms homotrimers that protrude from the lipid envelope. It is responsible for viral entry into the host cells by binding to the host receptor via receptor-binding domain (RBD). Tai et al showed that RBD protein interacts strongly with the human ACE2 receptors.[6] It is a highly conserved region within the viral spike protein and serves as the most important target for the development of an effective SARS-CoV-2 vaccine.[2,3,6] Various vaccine strategies are being applied across the globe to develop affordable and effective vaccine that could end the SARS-CoV-2 emergency.

Currently, vaccines with new technologies are being employed to handle several unresolved issues. Fast-tracked global efforts led to the development of more than 300 vaccine candidates within a span of 15 months of which over 40 are going clinical evaluation in different parts of the world. The lipid nanoparticle based mRNA vaccine (Moderna), viral vector (Oxford AstraZeneca), inactivated vaccine (Bharat Biotech), DNA based vaccine (Inovio Pharmaceuticals) and subunit vaccine (Novavax) being some of the notable vaccines being used to protect people from infection.[7] Major limitations for mass immunization like vaccine storage and ethical concerns regarding equitable distribution still loom the pandemic. The candidate front runners based on DNA and RNA are for the first time being applied in clinic and the long-term efficacy and safety data will only be available in the following years. Moreover, majority of these cannot be safely administered to high-risk individuals like those with weakened

Abbreviation: RBD, Receptor-binding domain; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; COVID19, coronavirus disease of 2019; PLA, poly lactide; PLGA, poly lactide-co-glycolide; WHO, World Health Organization; PHEIC, public health emergency of international concern; ACE2, angiotensin-converting enzyme 2.

* Corresponding authors.
E-mail addresses: rahul74@nii.ac.in (R. Ahuja), amulya11panda@gmail.com (A.K. Panda).
[1] Current Address: Panacea Biotec Limited, New Delhi-110044, India

https://doi.org/10.1016/j.xphs.2023.01.024
0022-3549/

immune system or elderly with comorbidities due to the increased risk of severe complications. The waning of immunity is a crucial concern with many proposing to jab the population with a third dose of vaccines.[8,9] This poses the risk of diverting the already limited vaccine supplies from people without any primary vaccination especially in middle- and low-income groups. This can further prolong the pandemic having pernicious impact on people already suffering from various other socio economic factors.[8,10,11] For example, Israel reported an increase in the number of cases among people who were vaccinated early in the pandemic rather than who were vaccinated later.[12]

New generation protein vaccines are by far the safest and most economical approach to eliminate SARS-CoV-2 disease. These are based on specific antigenic parts like purified proteins rather than the whole pathogen. This confers them with excellent safety profile and minimal side effects. Further, they do not involve use of any unsafe chemicals or danger of genetic integration. Most of the vaccines in various clinical phases today are protein based. However, these are weakly immunogenic and require the use of adjuvants and multiple doses to mount an effective antibody response.

Adjuvants range from aluminium salts to delivery systems like microparticles, liposomes, oil in water emulsions as well as various other substances like nucleic acid, cytokines, peptides, hormones, lipids or microbial products. Alum is the oldest and most widely used adjuvant in vaccines. The precedence of the particulate vaccine delivery system has only recently been recognized and is being strategically employed in vaccine development. A number of particulate delivery systems have been evaluated for antigen delivery like liposome, virosomes, poly lactide (PLA) and poly lactide-co-glycolide (PLGA) particles. Particles made of synthetic polymers like PLA and PLGA are the most favourable antigen delivery vehicle. These biodegradable polymers poly lactic acid (PLA) or poly lactide-co-glycolides (PLGA) have medically been in use as suture materials and diverse controlled release formulations.[13-15]

Vaccine delivery using PLA/PLGA polymeric particulate systems offer many advantages. The antigen release profile can be adjusted by using polymers of different molecular weight or different sized particles. This can be used to develop a single dose formulation mimicking the multidose vaccination regimen.[16,17] Vaccine formulated as particles in powder form, offer improved stability during storage and protect the antigens from degradation by cellular enzymes. This can be helpful in many of the developing countries where even the vaccine preventable diseases are way behind the global immunization target.[17-19] According to WHO, about 2.5 million children aged less 5 lose their lives from vaccine-preventable diseases across the globe. Further, with interruptions in vaccination programmes due to COVID-19, risk of resurgence in vaccine preventable diseases occur in at least 80 million children under the age of one. This is largely attributed to the requirement of booster doses owing to poor immunogenicity of vaccines. Polymer particles offer the possibility of entrapping multiple antigens, dose reduction, different size and additional adjuvant to induce effective immune response.

The present investigation involves the formulation of PLA polymer based microparticles entrapping protein subunit vaccine candidate, RBD of SARS-CoV-2. These microparticles show augmented immunogenicity, improved temperature stability and sustained antibody responses compared to alum adjuvanted RBD from single point immunization.

Materials

Poly-lactide (PLA, PURASORB) (45 kDa), Corbion Amsterdam, Netherlands; Alum (2% w/v alhydrogel), BrenntaG, Germany; 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate, eBioscience (Invitrogen, USA) were purchased. The RBD expressing bacteria was ordered from Dr. KPC Bioinnovations and Diagnostics (A group company under 'The KPC Group, California'), West Bengal, India. Dichloromethane (DCM), acetonitrile and sodium chloride were ordered from Merck, India. Mouse serum albumin, mannitol, polyvinyl alcohol (PVA), sodium bicarbonate, indoleacetic acid (IAA), Tween-20, sucrose, ammonium bicarbonate, o-phenylenediamine dihydrochloride (OPD) from Sigma-Aldrich, USA. Bicinchoninic acid (BCA) assay kit was purchased from Pierce, USA. SARS-CoV-2 (COVID-19) Spike RBD antibody from GeneTex, California, USA. Mouse anti-Rabbit IgG-HRP antibody from Santa Cruz Biotechnology, Texas, USA. Sequencing Grade Trypsin was purchased from Promega, USA. Emulsification steps were performed using BANDELIN SONOPLUS homogenizer (HD2200, Germany) or Heidolph SilentCrusher M (Germany). Freeze Dryer was from ilShinBioBase, South Korea. All other reagents were procured from local suppliers unless mentioned otherwise. All glassware were from Borosil, India. All buffers and solutions were prepared in Milli Q grade water.

Methods

Characterization of Refolded RBD by Mass Spectroscopy and Western Blotting

The protein was purified according as described previously from *E. coli* and subsequently characterized.[15] RBD was resolved on SDS-PAGE and in gel digestion was performed to prepare the sample for mass spectrometry. Followed by trypsinization for 12-16 hours at 37 degree Celsius, the supernatant containing peptides were transferred to fresh tube and concentrated to a volume of 10 μl using CentriVap Benchtop Vacuum Concentrator (Labconco, USA). The peptide solution was then desalted using C4 ZipTip (Merck Millipore, USA) and injected to a LC MS/MS system (Thermo LTQ Orbitrap Velos). The mass spectrum was acquired and analysed using Thermo Proteome Discoverer 1.3 software.

The purified RBD (0.1-1 μg) was resolved on a SDS-PAGE. A prestained marker was also ran along to confirm the transfer off proteins. The gel was then proceeded for western blotting. Millipore Immobilon P polyvinylidene fluoride (PVDF) membrane was used to blot the protein. The transfer was then carried out in transfer buffer containing 25 mM Tris, 192 mM glycine and 20 % methanol at 30 mV overnight at 4-8 degree Celsius for 12-16 hours. The membrane was blocked in 3 % BSA for 2 hours at 37 degree Celsius. The membrane was washed (twice) with Wash Buffer (0.1% Tween 20 in PBS) and incubated with 1:5000 dilution of SARS-CoV-2 RBD antibody at 4 degree Celsius for 12-16 hours. The membrane was again washed with washing buffer twice and anti-mouse HRP conjugated secondary antibody (dilution 1:10000) was added for incubation for 1 hour at 37 degree Celsius. The blot was then developed using chromogenic substrate 3,3′-diaminobenzidine (DAB).

Formulation of Polymer Microparticles Entrapping RBD of SARS-CoV-2

Recombinant SARS-CoV-2 RBD entrapped biodegradable microparticles were formulated using the double emulsion solvent evaporation method as described earlier.[20] The details of formulation parameters are presented in Table 1. RBD (3% w/v) was dissolved in internal aqueous phase (IAP) along with mouse serum albumin (MSA) and sucrose (2.5:10) total volume being 100 μl. IAP was subsequently added in a drop wise manner to organic phase (OP) comprising of 50 mg/ml PLA (4 ml) under sonication by BANDELIN Sonifier, Germany (30 % power, 40 duty cycles, 1 min). The resulting primary emulsion (W1/O) was then added drop by drop to 1 % PVA (30-70 kDa) and 10 % sucrose [external aqueous phase (EAP)] at 10000 rpm (Silent Crusher M, Heidolph, Germany) leading to secondary emulsion (W1/O/W2). The formulation was stirred for 7-8 hours, 800 rpm for the evaporation of DCM. The microparticles were recovered by

Table 1
Parameters used RBD microparticle formulation.

Formulation	IAP	OP	EAP	IAP:OP	OP:EAP
Microparticles	3 % RBD (w/v) + 2.5 % MSA (w/v) + + 10 % Sucrose (w/v)	50 mg/ml PLA in DCM	1 % PVA (w/v) + 10 % Sucrose (w/v)	1:40	1:4

IAP: Internal aqueous phase, OP: Organic Phase, EAP: External aqueous phase, MSA: Mouse serum albumin, DCM: Dichloromethane, PLA: Poly (d,l) lactic acid, PVA: Polyvinyl alcohol

centrifugation (12000 rpm, 4 degrees Celsius, 30 minutes) and freeze dried (ilShinBioBase Freeze Dryer, South Korea).

Dynamic Light Scattering and Zeta Potential of RBD Entrapped Polymer Particles

RBD polymer microparticles were characterized by Zetasizer Nano ZS (Malvern Instruments Limited, U.K.) for estimation of size, zeta potential as well as polydispersity index. For this, around 1-2 mg of RBD microparticles in 1 ml of water were placed in sizing plastic cuvette (size) and folded capillary cell (zeta potential) and recorded using Zetasizer Software (Version 7.12). Three measurements were recorded and average calculated.

Scanning Electron Microscopy of RBD Entrapped Polymer Particles

RBD entrapped microparticles were further characterized for true size, shape as well as surface properties with the help of scanning electron microscopy. About 10 μl of diluted suspension of RBD microparticles placed over the coverslip in a monolayer. Air dried cover slip containing the dried and adherent particles was coated with gold palladium (EMS SC7620 Mini Sputter Coater). Images then acquired (ZEISS EVO LS10 Tokyo, Japan) and analyzed using Smart SEM digital software.

Estimation of RBD Entrapped Polymer Particles

The RBD content of polymer microparticles was estimated using Bicinchoninic acid assay. About 4-5 mg of particles in acetonitrile (1 ml) precipitated the RBD recoverable as pellet after centrifugation at 10000 rpm. The protein was dissolved in 1% sodium dodecyl sulphate (SDS) and estimated as per kit instructions with the help of BSA standard (1.5 μg/ml – 100 μg/ml).

RBD content of microparticles was calculated as:

Antigen load = RBD per mg of microparticles.

$$= \frac{\text{Concentration x Dilution}}{\text{1000x Weight of particles}} \text{x Resuspension Volume}$$

Immunological Evaluation of RBD Entrapped Polymer Particles from Single Point Immunization

Inbred BALB/c mice (6-8 weeks old) were used to carry out all the animal experiments. The animals were housed following the guidelines of institutional animal ethics committee of National Institute of Immunology (IAEC Number 555/20). All immunization were carried out by intramuscular route. Pre-bleed collected one week before staring the experiment was used as control. Table 2 shows the details of different formulations used. The required particles were accurately weighed and aseptically dissolved in phosphate buffered saline just prior to immunization. For the alum adjuvanted group, alum binding with RBD was carried out for a duration of 2 hours at room temperature with the help of a rocker shaker (Table S1). 20 μg of alum per 10 μg of RBD was used. All the groups were immunized at 10 μg RBD per mice. To assess the stability of microparticle formulations, microparticles stored at 25 and 37 degrees Celsius were used for immunogenicity comparison with those stored in cold chain. To further evaluate the antibody recall response against RBD by single point immunization, mice were boosted with 2 μg of soluble RBD after 120 days. The mice were bled every 2 weeks until the end of study and separated serum stored at -20 degree Celsius.

Table 2
RBD formulations used for immunization studies.

Serial No.	Group	Formulation used for immunization
1	RBD only	Soluble RBD only
2	RBD + alum	Alum adjuvanted RBD
3	RBD MP4	RBD microparticles stored in cold chain
4	RBD MP25	RBD microparticles stored at 25 degree Celsius
5	RBD MP37	RBD microparticles stored at 37 degree Celsius

Anti-RBD Total IgG Antibody Estimation

Total IgG was estimated following a similar protocol with slight modification.[18,21] High binding ELISA plates coated with 10 μg/ml of RBD (50 μl) in coating buffer (carbonate buffer, pH 9.6-10.6) were incubated at 4 degrees Celsius overnight. The plates were blocked with 1 % BSA (bovine serum albumin) at 37 degrees Celsius for 2 hours. The plates were washed twice with 0.05 % Tween 20 in PBS (Wash Buffer). Next, serially diluted mice sera in PBS was added and incubated at 4 degrees Celsius overnight. Spike RBD monoclonal antibody (Genetix) was used as standard (5 ng/ml – 150 ng/ml). Subsequently, plates were rinsed with Wash Buffer twice. HRP-conjugated total IgG antibody (dilution 1:10000) was added to the plate and incubated for 1 hour at 37 degree Celsius. Plates were rinsed with Wash Buffer twice and then developed with 3,3′,5,5′-Tetramethylbenzidine (TMB substrate, eBiosciences, USA). The addition of TMB was carried out in dark and Stop solution containing 1.5 N H_2SO_4 was used to stop the reaction and OD value was measured at 450 nm (Bio-Tek spectrophotometer, Vermont, USA).

Statistical Analysis

Different formulations were administered at 5 mice in each group. ELISA was used for determining the RBD antibody generated and OD was measured at a wavelength of 490 nm. Each sample measurement denotes a mice and OD denotes the group mean. Statistical significance of group mean and standard deviation (SD) was assessed by Two –Way Analysis of Variance (Two-way ANOVA). Tukey-Kramer test for multiple comparison, recommended for groups with n >3, was used for comparison of groups. The test was only implemented if the p value was less than 0.5. Confidence interval of 95 % was used for performing all the tests. GraphPad Prism 8 (GraphPad Software Inc., USA) was used for performing the statistical comparison for all the groups tested.

Results and Discussion

Characterization of Refolded RBD by Mass Spectroscopy and Western Blotting

Recombinant RBD was purified as described earlier.[15] The sequence of purified RBD protein was analyzed by mass spectroscopy. The data acquired was analyzed using Thermo Proteome Discoverer software. The mapping of the acquired peptide spectra

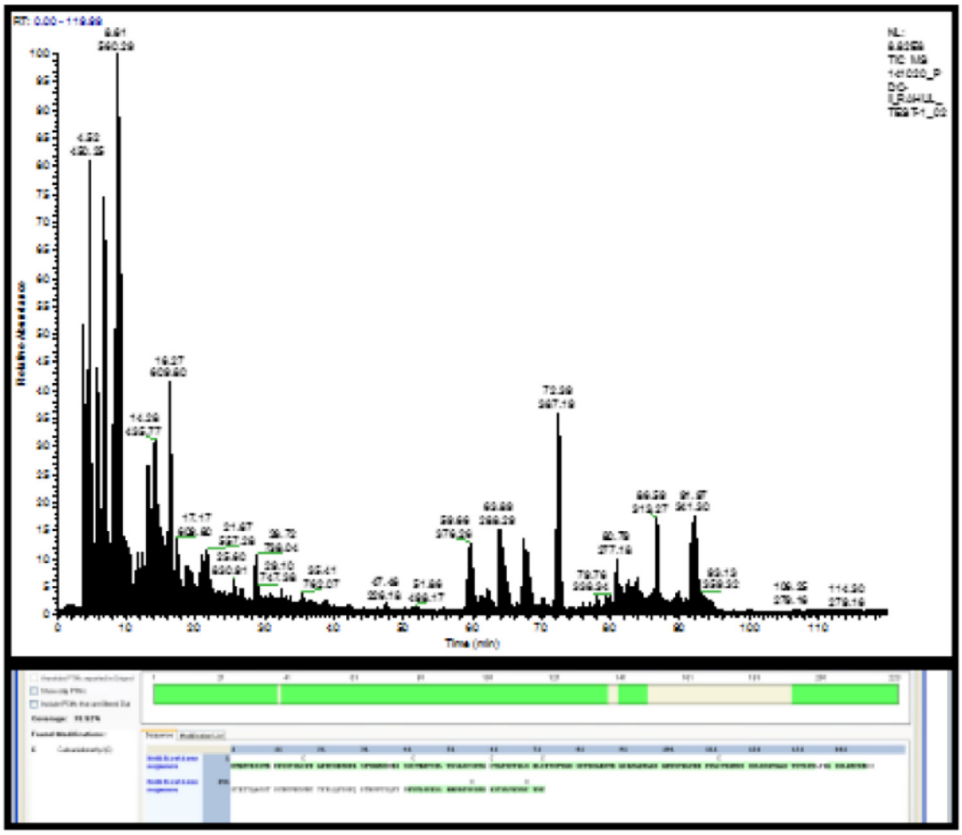

Figure 1. Sequence identification of purified RBD. The peptide spectra of RBD was acquired using Orbitrap Velos mass spectrometer. Thermo Proteome Discoverer Software was then queried against SARS CoV-2 database. A sequence coverage of 78.92 % was obtained.

showed 78.92 % sequence coverage with SARS-CoV-2 RBD (Fig. 1). This confirmed the authenticity of purified SARS-CoV-2 RBD. The protein was blotted onto a nitrocellulose membrane and stained with the commercially available SARS-CoV-2 RBD antibody (against glycosylated RBD). 3,3′ diaminobenzidine (DAB) was used to develop the blot and gave clear bands at the respective molecular weight (Fig. 2). Thus, the presence of RBD was confirmed and reactivity with commercially available antibody was indicative of its epitope retention.

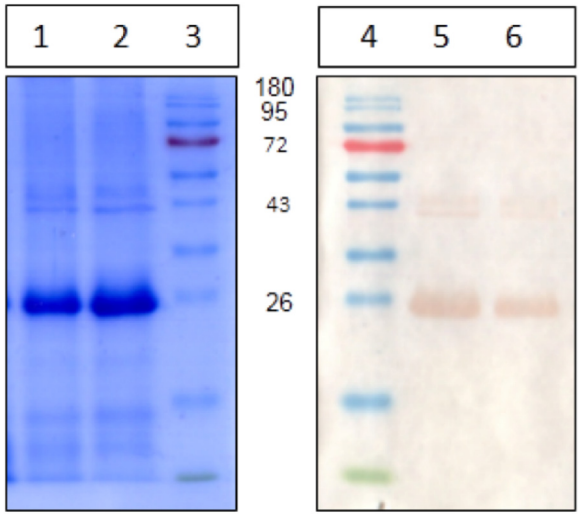

Figure 2. Immunoblot of purified RBD using commercially available antibody. Lane 1, 2 represent the resolved RBD (two different batches) on SDS-PAGE, Lane 5, 6 represent the immunoblot of RBD. Lane 3, 4 represent the pre stained ladder.

Formulation and Characterization of Microparticles Entrapping RBD of SARS-CoV-2

Double emulsion solvent evaporation method was used to entrap the RBD in polymer particles. Polylactic acid (PLA), a biodegradable and biocompatible polymer was used in the study. Mouse serum albumin in the internal aqueous phase protected the protein from dichloromethane induced denaturation.[22] Sucrose was included at 10 % w/v in both internal aqueous phase and external aqueous phase to balance the osmolality and as a lyoprotectant. Microparticles made of polylactic acid entrapping RBD were successfully formulated. The protein content of the particles was estimated by bicinchoninic acid assay and load of RBD was estimated to be 5.0 μg per mg of polymer particles.

Characterization of RBD Entrapped Polymer Particles

The RBD entrapped microparticles were characterized for size and zeta potential using Nano ZS ZetaSizer. Microparticles entrapping RBD were assessed for size using dynamic light scattering and were estimated to be of size 3380 nm with a polydispersity index of 0.052. Further, the particles were characterized for the charge present on their surface. The electrophoretic mobility measurement was used to estimate the zeta potential for RBD microparticles which was -27.6 mV. A potential more than +10 mV or -10mV is an indication of stable particles suspension.

Morphological Characterization of Polymer Particles Entrapping RBD

Scanning electron microscopy was used to further characterize the RBD microparticles (Fig. 3). Multiple regions were imaged and

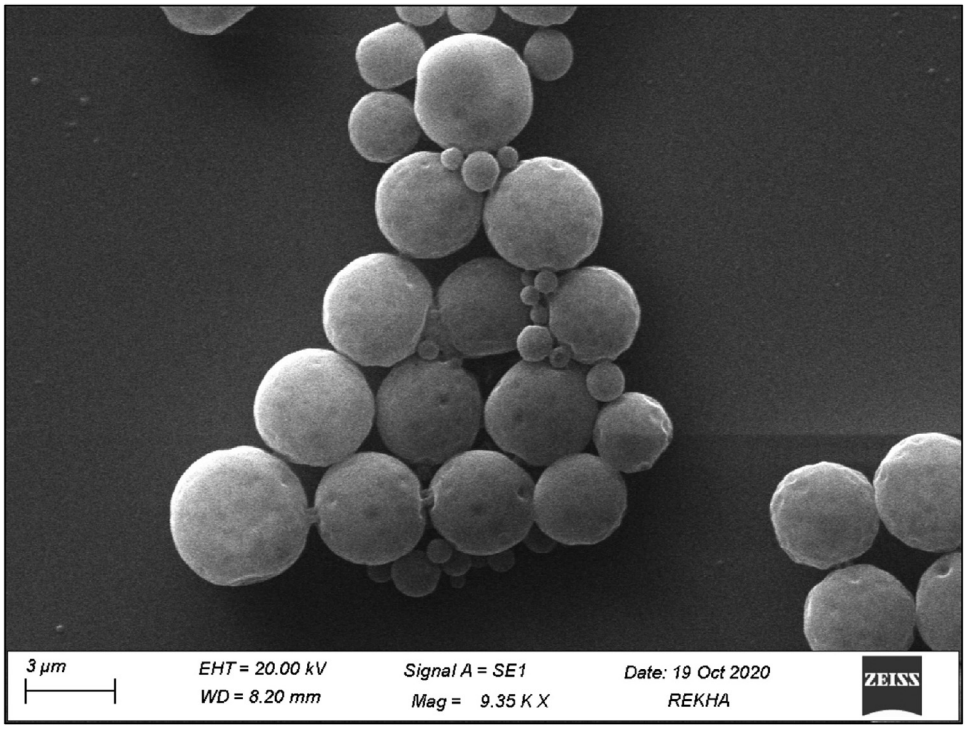

Figure 3. Representative scanning electron microscopy of RBD entrapped microparticles.

exhibited smooth, spherical morphology of RBD microparticles. Size of the particles was estimated to be 4.1 ± 0.32 μm. They all showed a spherical shape with clefts of 200 - 300 nm indicative of porous channels beneath. The details of prepared polymer particles entrapping RBD are presented in Table 3.

Immunological Evaluation of RBD Entrapped polymer particles

For single dose immunogenicity evaluation, 6-8 week old BALB/c mice were immunized intramuscularly with a dose of 10 μg RBD per mice in different groups: a. soluble RBD only, b. alum adjuvanted RBD and c. microparticles entrapping RBD. Serum was analysed by ELISA. Enhanced antibody response was observed both with the alum adjuvanted formulation as well as microparticles. The highest antibody response was observed with microparticles entrapped RBD (Fig. 4). Microparticles showed peak antibody response at about day 15-21 whereas alum adjuvanted formulation showed a peak day 15 after which it rapidly declined. Though, microparticles showed a modest decline by day 30, it was noted that a second peak was observed at about day 73 after which the antibody response started to decline. The second peak could be due to the release of protein due to bulk hydrolysis of polymer. For the alum adjuvanted RBD formulation, the antibody response rapidly declined by day 30 whereas for microparticles the response was sustained till 4 months. Thus, microparticles were able to sustain the antibody compared to alum adjuvanted RBD. The peak response achieved by microparticles was 60 % more than by alum adjuvanted protein and sustained at a higher level for four months. It is of interest to note that higher and better sustained antibody response with RBD microparticles was observed without the use of alum adjuvant.

This could be explained by the fact that microparticles act like depot and continuously release the protein for activating immune system.[23] Antibody titres from the microparticle formulation were sustained in comparison to alum adsorbed RBD. Thus, the use of microparticle formulations not only improved the immunogenicity of RBD but also gave sustained antibody response from single point administration. This clearly indicated the benefit of using microparticles as delivery system for immunization.

Immunogenicity Study of RBD Entrapping Polymer Microparticles Stored at Different Temperatures

Vaccines are most often limited in their access to low- and middle-income parts of the world due to the requirement of cold chain storage. These typically require the greatest number of vaccines especially in case of infectious disease like COVID due to large population concentration. Vaccines with improved temperature stability can

Table 3
Characterization of RBD entrapped polymer particles.

Formulation	Particle size (nm)	Zeta potential (mV)	PDI	Load (μg/mg)
RBD MP	4.1 ± 0.32 μm	-27.6	0.052	5.0 μg

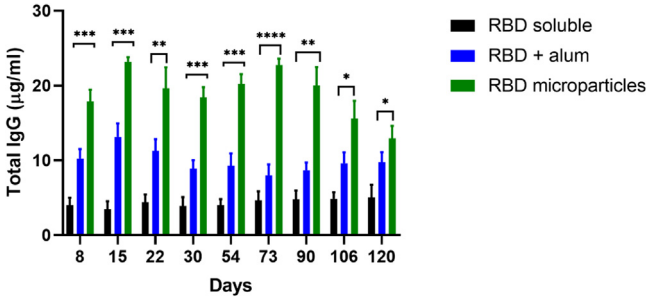

Figure 4. Anti-RBD response elicited by immunization of RBD entrapped polymeric particles at single dose. BALB/c mice (n=5) injected with RBD entrapped microparticles (▲), RBD adjuvanted with alum (■) and soluble RBD (●).

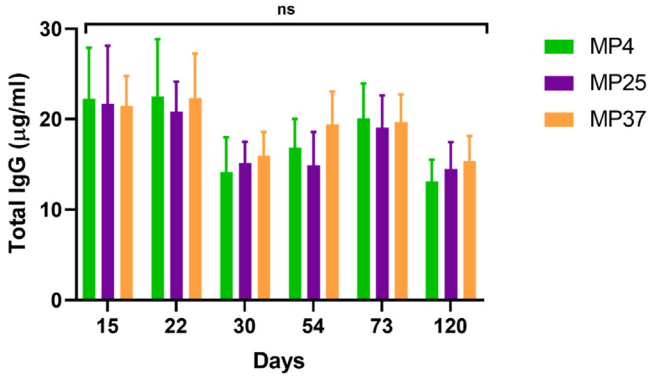

Figure 5. Anti-RBD antibody response elicited by immunization of RBD entrapped microparticles stored at various temperatures. Five mice (BALB/c) were injected with RBD microparticles at 4 degree Celsius (—●—), 25 degree Celsius (—■—) and 37 degree Celsius (—▲—).

greatly help increase access, lower the cost and save more lives.[24] To assess the stability, microparticles entrapping RBD were stored at 4, 25 and 37 degrees Celsius for a period of one month and then used for immunization.

All of the RBD microparticle formulations elicited antibody response (Fig. 5). For all the formulations, a peak was observed at about day 15-21. All the groups, similar to the previous immunization, showed a second peak at about day 70 after which the response started to decline. However, no significant difference in antibody titres was observed for any individual time point tested. Thus, no considerable loss in immunogenicity could be observed for the microparticle formulation when stored at increased temperatures in comparison to cold chain. This has wide scale implications for increasing the coverage, reducing cost and preventing the loss of vaccines due to accidental exposure to high temperatures especially in less developed nations with improper cold chain facilities.

Long Lasting Memory Evaluation of RBD Entrapped Polymer Particles

It was of interest to check whether single point immunization could also generate memory antibody response. For this, the immunized mice were challenged with 2 μg of soluble RBD after four months. The group immunized with soluble RBD could not provoke any recall response whereas both the other groups elicited secondary antibody response (Fig. 6). The degree of secondary antibody response generated was different for the microparticle formulation and alum adjuvanted RBD group. The microparticles elicited higher recall response than the alum adjuvanted RBD. For both, the alum adjuvanted and microparticle formulations the peak response was achieved at about day 21 post boost. However, the peak secondary

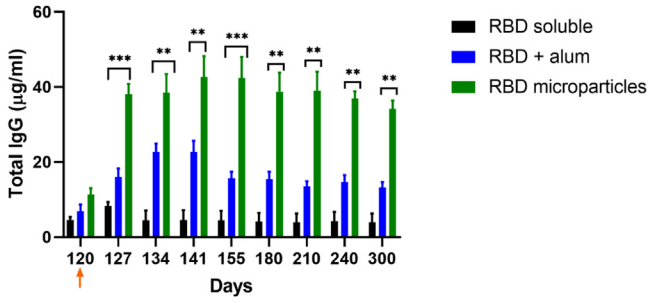

Figure 6. Anti-RBD recall response elicited by immunization of RBD entrapped polymeric particles. Five mice (BALB/c) were jabbed with RBD microparticles (—▲—), RBD adjuvanted with alum (—■—) and soluble RBD (—●—). All the mice were boosted after 120 days with 1/5th primary dose of RBD.

response for the microparticle was 1.6-fold the peak secondary response achieved by alum adjuvanted RBD. For alum adjuvanted group the recall response declined rapidly whereas the microparticle formulation sustained the antibody response till the end of study (six months). Thus, microparticulate formulation of RBD generated improved and more sustained antibody response compared to alum adjuvanted RBD.

It is of interest to note that such long-term recall response of microparticles was accomplished without the use of alum adjuvant from single point administration. These results suggest that polymer microparticle formulation of RBD not only enhanced the primary antibody response but also generate long lasting and enhanced recall response after single point vaccination. Polymer particle entrapped RBD provides an opportunity to develop single dose immunization for corona vaccine than multiple boosters. Nevertheless, it is worthwhile to mention that this study looked at B cell generated antibody response while T cell responses are also important in counteracting the virus. Further, we were unable to perform the neutralization assay in this particular study which is a better indicator of protective potential. We consider these as the limitation of our study.

Conclusion

Vaccines based on conserved SARS-CoV-2 protein antigens are a promising and safe strategy for vaccine development. They are, however, limited by poor immunogenicity and require the use of adjuvants along with multiple doses. In this study, RBD of SARS-CoV-2 was entrapped in microparticles and evaluated for immunogenicity, temperature stability and recall response from single dose administration. The *in vivo* studies demonstrate that single dose administration of microparticles entrapping RBD mounted increased antibody response compared to alum. Moreover, these particles could be stored safely at 37 degrees for one month without any appreciable loss of immunogenicity. The antibody response elicited was sustained for a longer period of time compared to alum. The memory antibody response elicited by microparticles were better and sustained till 6 months post challenge. Thus, microparticles entrapping RBD not only enhanced the immunogenicity and conferred sustained memory antibody response from single point administration but also showed better temperature stability. These dried thermostable microparticles can provide a wider access of vaccines to resource limited settings, reduce cost and help in achieving a faster end of the pandemic.

Data availability

All data used or analyzed are available from the corresponding authors upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Rahul Ahuja: Conceptualization, Validation, Visualization, Data curation, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sudeepa Srichandan:** Investigation, Formal analysis, Validation, Data curation. **Jairam Meena:** Validation, Data curation, Writing – review & editing. **Bichitra Kumar Biswal:** Resources, Writing – review & editing. **Amulya K. Panda:** Funding acquisition, Supervision, Conceptualization, Methodology, Visualization, Validation, Resources, Data curation, Formal analysis, Writing – review & editing.

Acknowledgements

The authors thank the core grant of National Institute of Immunology to AKP for the funding and DBT-JRF Programme of Department of Biotechnology for support of RA doctoral research.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2023.01.024.

References

1. Worldometer. *Coronavirus pandemic*. 2021 July 22, 2021]; Available from: https://www.worldometers.info/coronavirus/.
2. Wrapp D, Wang N. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*. 2020;367(6483):1260–1263.
3. Chams N, et al. COVID-19: a multidisciplinary review. *Front Public Health*. 2020;8:383.
4. Frederiksen LSF, et al. The long road toward COVID-19 herd immunity: vaccine platform technologies and mass immunization strategies. *Front Immunol*. 2020;11:1817.
5. Korber B, et al. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. *Cell*. 2020;182(4):812–827. e19.
6. Tai W, et al. Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: implication for development of RBD protein as a viral attachment inhibitor and vaccine. *Cell Mol Immunol*. 2020;17(6):613–620.
7. Jeyanathan M, et al. Immunological considerations for COVID-19 vaccine strategies. *Nat Rev Immunol*. 2020;20(10):615–632.
8. Rai D. Lessons from Israel: how long can the two doses protect us from coronavirus? *India Today*. New Delhi: Living Media India Limited; 2021.
9. Sumant Sen JN. Israel's Recent COVID-19 Spike Explained *in* The Hindu. Chennai, Tamil Nadu, India: N Ravi, The Hindu Group; 2021.
10. Scott J, Richterman A, Cevik M. Covid-19 vaccination: evidence of waning immunity is overstated. *BMJ*. 2021;374:n2320.
11. Levin EG, et al. Waning immune humoral response to BNT162b2 Covid-19 vaccine over 6 months. *N Engl J Med*. 2021.
12. Estrin D. Highly Vaccinated Israel Is Seeing A Dramatic Surge In New COVID Cases. Here's Why, *in* All Things Considered. Washington, D.C, United States: National Public Radio; 2021.
13. Frazza EJ, Schmitt EE. A new absorbable suture. *J Biomed Mater Res*. 1971;5(2):43–58.
14. O'Hagan DT, Singh M. Microparticles as vaccine adjuvants and delivery systems. *Expert Rev Vaccines*. 2003;2(2):269–283.
15. Meena J, et al. RBD decorated PLA nanoparticle admixture with aluminum hydroxide elicit robust and long lasting immune response against SARS-CoV-2. *Eur J Pharm Biopharm*. 2022;176:43–53.
16. Katare YK, Panda AK. Immunogenicity and lower dose requirement of polymer entrapped tetanus toxoid co-administered with alum. *Vaccine*. 2006;24(17):3599–3608.
17. Katare YK, Panda AK. Influences of excipients on in vitro release and in vivo performance of tetanus toxoid loaded polymer particles. *Eur J Pharm Sci*. 2006;28(3):179–188.
18. Kanchan V, Katare YK, Panda AK. Role of alum in improving the immunogenicity of biodegradable polymer particle entrapped antigens. *Eur J Pharm Sci*. 2009;38(1):18–28.
19. Kanchan V, Panda AK. Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. *Biomaterials*. 2007;28(35):5344–5357.
20. Ahuja R, et al. Microparticles entrapping pneumococcal protein SP0845 show improved immunogenicity and temperature stability. *Int J Biol Macromol*. 2022;203:661–670.
21. Tan HX, et al. Immunogenicity of prime-boost protein subunit vaccine strategies against SARS-CoV-2 in mice and macaques. *Nat Commun*. 2021;12(1):1403.
22. Raghuvanshi RS, et al. Stabilization of dichloromethane-induced protein denaturation during microencapsulation. *Pharm Dev Technol*. 1998;3(2):269–276.
23. Kanchan V, Katare YK, Panda AK. Memory antibody response from antigen loaded polymer particles and the effect of antigen release kinetics. *Biomaterials*. 2009;30(27):4763–4776.
24. Dumpa N, et al. Stability of vaccines. *AAPS PharmSciTech*. 2019;20(2):42.