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RESEARCH PAPER

# Nucleic Acid Aptamers as Stabilizers of Proteins: The Stability of Tetanus Toxoid

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## ABSTRACT

***Purpose*** Exposure of tetanus toxoid to moisture leads to its aggregation and reduction of potency. The aim of this work was to use SELEX (systematic evolution of ligands by exponential enrichment) protocol and select aptamers which recognize tetanus toxoid ($M_r$ ~150 kDa) with high affinity.

***Methods*** Colyophilized preparations of tetanus toxoid and specific aptamers were encapsulated in PLGA microspheres and sustained release of the antigen was observed up to 55 days using different techniques.

***Results*** The total protein released was between 40–55% (24–45% residual antigenicity) in the presence of the aptamers as compared to 25% (11% residual antigenicity) for the antigen alone. We show that instead of inhibiting absorption of moisture, the aptamers blocked the protein unfolding upon absorption of moisture, inhibiting the initiation of aggregation. When exposed to accelerated storage conditions, some of the RNA sequences were able to inhibit moisture-induced aggregation *in vitro* and retain antigenicity of tetanus toxoid.

***Conclusions*** Nucleic acid aptamers represent a novel class of protein stabilizers which stabilize the protein by interacting directly with it. This mechanism is unlike that of small molecules which alter the medium properties and hence depend on the stress condition a protein is exposed to.

**KEY WORDS** aptamers · controlled drug release · protein stability · tetanus toxoid · vaccine

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## INTRODUCTION

The World Health Organization (WHO) estimates that the current cost of immunization programmes in developing countries far exceeds the actual cost of the vaccines. Field reports from concerned organizations indicate an unacceptable level of vaccine coverage in 'priority countries' (1–4). A major cause of this has been identified as the instability of the protein during its storage and transport, both in the raw material and finished product forms (3,5). Similar to other solid protein drugs (6–8), tetanus toxoid can also absorb moisture and undergo denaturation and aggregation during storage and transport (9,10). The presence of formaldehyde in the attenuated toxin may lead to the formation of Schiff bases by reaction with amine groups of proteins, leading to further crosslinking (11,12). This results in loss of the starting product and an overall increase in the cost of vaccine production. For immunization against tetanus, the vaccine is required to be delivered in multiple doses, where the initial dose is followed by booster doses at defined time intervals. In many cases, the infants are not brought back to the health centres following the first dose. This gives rise to an additional challenge to the immunization programme. One suggestion to overcome this problem has been the proposal to use single dose controlled release devices. These would slow down the release of the toxoid over a period of time, thus partially eliminating the need for patient compliance. Poly (lactide-co-glycolide) (PLGA) based controlled release devices have been proposed to reduce the dosing frequency of vaccines, resulting in higher vaccine coverage (12,13). Such devices face the problem of protein aggregation following the exposure of vaccines to moisture during rehydration, at the time of release in the body (14,15). Water sorption capacity of most of the polymers/hydrogels which make up such devices is very high and this water may also interact with the entrapped protein leading to deleterious

results. This aggregation of the protein (antigen) leads to incomplete release and thus reduced efficacy of vaccine. These problems have been addressed by modifying the toxoid by neutralizing the reactive nucleophiles generated during formalinization of the toxin and by addition of low-molecular-weight excipients like histidine, lysine, sorbitol, trehalose, magnesium carbonate, *etc.* (15). These classical ways of stabilizing tetanus toxoid are not sufficient to tackle a wide variety of damaging conditions. Batches of vaccines need to be discarded, leading to under-immunization of a large section of the population. Thus, the disease remains prevalent in developing countries where stability of the protein is a commonly encountered problem.

Aptamers are single-stranded nucleic acids which recognize their targets specifically with high affinity. Aptamers have been selected against a variety of targets, including proteins such as growth and clotting factors, cell-surface proteins, cancer cells, small molecules such as nucleotides, antibiotics, organic dyes, cofactors, sugars, amino acids, *etc.* (16–20). Aptamers are referred to as "chemical antibodies", the advantage being that their synthesis, being an *in vitro* process, is more reproducible than that of antibodies which are produced in biological systems (21–23). They can also be selected against toxic and/or self targets. The specificity of aptamers for their targets, and their non-toxic and non-immunogenic nature (18–20), make them attractive candidates for inhibition of protein aggregation. Since aggregation of tetanus toxoid inside PLGA microspheres has been identified as one of the main reasons for the incomplete release of the antigen, in the present study, we have selected specific aptamers which bind to tetanus toxoid with high affinity. We have studied the effect of these RNA aptamers on the aggregation of tetanus toxoid encapsulated in PLGA microspheres and have attempted to elucidate the mechanism of stabilization of the protein.

## MATERIALS AND METHODS

### Materials

Tetanus toxoid (2550 Lf/unit with antigenic purity of 1572 Lf $mg^{-1}$ of protein nitrogen) was obtained as a gift from Shantha Biotechnics Ltd., Hyderabad, India. Cyanogen-bromide activated agarose resin, ribonuclease A (105 kunitz unit $mg^{-1}$), bovine serum albumin (BSA, fraction V), deoxyribonucleotides (dNTPs), ribonucleotides (rNTPs), agarose, ethidium bromide, Luria-Bertani broth, agar and ampicillin were purchased from Sigma-Aldrich, Bangalore, India. Yeast inorganic pyrophosphatase (0.1 U $\mu l^{-1}$), RNase-free DNase I (1 U $\mu l^{-1}$) and T7 RNA polymerase (20 U $\mu l^{-1}$) were purchased from Fermentas Inc., Maryland, USA. RNase inhibitor (RNase OUT, 40 U $\mu l^{-1}$) was purchased from Invitrogen Corporation, California, USA. *Taq* DNA polymerase (5 U $\mu l^{-1}$), pGEM®-T Easy Vector kit, Wizard® SV Gel and PCR Clean-Up System and MMLV reverse transcriptase (200 U $\mu l^{-1}$) were obtained from Promega Corporation, Madison, USA. Fluorescein RNA labeling mix was obtained from Roche Applied Sciences, Mumbai, India. Mouse anti-tetanus toxoid monoclonal antibody (HYB 278-01), raised against full length formaldehyde inactivated tetanus toxoid, was purchased from Santa Cruz Biotechnology, Inc., California, USA. Tetanus hyper-immune globulin (human) (Tetglob®) (human polyclonal tetanus toxoid anti-serum) was a product of Bharat Vaccines and Serum Limited, Mumbai, India and was purchased from the local pharmacy. Goat anti-mouse horseradish peroxidase conjugated monoclonal antibody and tetramethyl benzidine/hydrogen peroxide substrate were obtained from Bangalore Genei, Banglore, India. Poly (lactide-co-glycolide) (PLGA) was obtained as a gift from PURAC Biochem, Gorichem, the Netherlands. All other reagents and chemicals used were of analytical grade or higher.

### Methods

#### *Synthesis of Degenerate RNA Libraries*

Two degenerate ssDNA libraries of 83 nt (5′-GATAAT ACGACTCACTATAGGGA TAGGATCCACAT CTACG$N_{20}$AAGCTTCGTCAAGTCTGCAGTAA-3′) and 97 nt (5′-AATGCTAATACGACTCAC TATAGGGAGAGAGAGACAGTCT$N_{37}$AAGCAAC GTCAACTCCAGAA-3′) were designed (labeled as T1 and T2, respectively) in the laboratory and synthesized by Sigma-Aldrich, Bangalore, India. T1 comprised of a 20 nt variable region whereas in T2, this region consisted of 37 nt. Both the sequences were flanked by a 40 nt constant region at 5′ end (which contained the sequence of T7 RNA polymerase promoter for *in vitro* transcription) and by 23 nt and 20 nt for T1 and T2, respectively, at the 3′ end. Amplification of the degenerate libraries was carried out by PCR (polymerase chain reaction) using a set of specific primers. For T1, the forward primer was 5′-GATAATACGACTCACTATAGGGATAGGATCC ACATCTACG-3′ and the reverse primer was 5′-TTACTGCAGACTTGACGAAGCTT-3′. For T2, the forward primer was 5′-AATGCTAATACGACTCACTA TAGGGAGAGAGAGACAGTCT-3′ and the reverse primer was 5′-TTCTGGAGTTGACGTTGCTT-3′. The amplified libraries were subjected to *in vitro* transcription. Following digestion with DNase I, the products were purified by denaturing polyacrylamide gel electrophoresis (8%, 8.3 M urea). RNA was extracted from the gel and precipitated using 70% (v $v^{-1}$) cold ethanol. The purified RNA was quantified spectrophotometrically at 260 nm and used as the starting RNA library for the selection procedure.

### *Selection of Tetanus Toxoid-Specific RNA Sequences*

Tetanus toxoid was immobilized on pre-washed cyanogen-bromide activated agarose resin as per published protocol (24). RNA pools, T1 and T2 (40 pmol each), were incubated separately with immobilized tetanus toxoid (40 pmol of protein) for 30 min in 100 µl of 50 mM Tris HCl buffer, pH 7.4 containing different concentrations of $MgCl_2$, *viz.* 2 mM, 3 mM or 4 mM. The supernatant was removed by centrifugation at 500 g for 5 min at 4°C. The matrix was washed four times with 200 µl of the respective selection buffer. Bound RNA was eluted by heating the suspension at 80°C for 3 min and recovered by centrifuging the heated suspension at 500 g for 5 min at 4°C. The partially enriched RNA pools were reverse transcribed to cDNA and amplified by PCR. The products obtained were used for subsequent rounds of selection as described above. Ten such cycles were carried out for each set of conditions.

### *Determination of Binding Affinity*

Dot blot assay was carried out to determine the binding affinities (measured as dissociation constants) of the enriched RNA pools obtained after ten cycles of SELEX for the protein. A constant concentration of fluorescein labeled RNA was incubated with increasing concentrations (1–1500 nM) of tetanus toxoid in the respective selection buffers. The samples were filtered through pre-wetted PVDF membrane (0.45 µm) using a 96-well vacuum filtration manifold (Whatman-Biometra, Goettingen, Germany). The retained protein-RNA complex was washed with 1000 µl of the respective selection buffer. The fluorescence intensity of the retained protein RNA complex was measured on Typhoon Trio (GE Healthcare, Uppsala, Sweden) in the fluorescence mode. The amount of retained fluorescein labeled RNA was quantified by Image Quant$^{TM}$ software (GE Healthcare, Uppsala, Sweden). The dissociation constants were determined by fitting the data into the Boltzmann's equation.

$$y = y_i + mx_i + \frac{y_f + mx_f}{1 + e^{\frac{x - x_0}{\tau}}}$$

where $y$ is the fluorescence intensity. Initial and final base lines are described by $y_i + mx_i$ and $y_f + mx_f$, respectively. $x_o$ denotes the concentration for 50% of maximal fluorescence (25). Dissociation constant ($K_d$) was calculated from the inflection point in the exponential part of the curve.

### *Synthesis of Monoclonal RNA Sequences*

The enriched RNA libraries were reverse-transcribed to generate the enriched DNA libraries, ligated to pGEM®-T Easy Vector and cloned into competent *E. coli* DH5α cells. The transformed cells were plated onto $LB_{amp}$ plates and incubated overnight at 37°C. Each plate had clones from a single enriched library. Fifty colonies were picked from each plate and grown overnight at 37°C in 10 ml $LB_{amp}$ culture media. Plasmid DNA was extracted by alkaline lysis method (26). PCR was carried out using the primers described above. The sizes of the insert sequences were confirmed by agarose gel electrophoresis. Each monoclonal DNA sequence was subjected to *in vitro* transcription to generate monoclonal RNA sequences.

### *Moisture-Induced Aggregation of Tetanus Toxoid*

Tetanus toxoid was incubated with individual RNA clones (2.5:1, w $w^{-1}$) for 1 h. The samples were then lyophilized and subjected to moisture-induced aggregation in a desiccator containing saturated solution of $K_2CrO_4$ (86% RH), as described earlier (10). After 3 days, the suspensions were centrifuged (16,000 g, 10 min) to separate the aggregated insoluble protein from the soluble fraction. The amount of protein precipitated was determined by subtracting the amount of protein present in the supernatant from that present in the starting sample, by the Bradford method (27), using bovine serum albumin as the standard protein.

### *Determination of Antigenicity of Tetanus Toxoid*

Antigenicity of total samples was determined by ELISA by following the reported protocol (14). Detection was carried out using mouse anti-tetanus toxoid monoclonal antibody (1:5000) as the primary antibody and HRP-conjugated anti-mouse antibody (1:3000) as the secondary antibody. The antigenicity of tetanus toxoid was detected using tetramethyl benzidine/$H_2O_2$ as the substrate for HRP. The colour formed was measured at 450 nm after terminating the reaction with 0.02 N $H_2SO_4$. Confirmatory ELISA was also carried out using the same procedure but with human polyclonal tetanus toxoid serum as the primary antibody and HRP-conjugated anti-human antibody as the secondary antibody.

### *Estimation of Water Content*

Tetanus toxoid (in the presence and absence of RNA sequences) was subjected to moisture-induced aggregation as described above (10). After 3 days, the samples were reconstituted in 1 ml of anhydrous methanol. A known amount of the sample was transferred to a potentiometric Karl Fisher titrator vessel (716 DMS, Metrohm) and the amount of water absorbed was determined by Karl Fischer titration. The values obtained were corrected for moisture content of anhydrous methanol, which was taken as the blank.

### *Preparation and Characterization of PLGA Microspheres*

Tetanus toxoid and colyophilized tetanus toxoid-RNA complexes (2.5:1, w $w^{-1}$) were encapsulated in poly (lactide-co-glycolide) (PLGA) microspheres by double emulsion/solvent evaporation method following protocols reported in literature (28,29). Microspheres were characterized by measurement of zeta potential, size and surface morphology. For estimation of zeta potential, microspheres were suspended in water and zeta potential was estimated by Zeta Sizer (Nano ZS, Malvern Instruments, Worcestershire, U.K.). Size and surface morphology of the microspheres were determined by scanning electron microscopy. Air-dried samples were gold coated and viewed under scanning electron microscope (JSM6100, JEOL Ltd., Tokyo, Japan).

### *Analysis of In Vitro Release of Tetanus Toxoid from Microspheres*

For *in vitro* release experiments, 50 mg of tetanus toxoid/tetanus toxoid-RNA complex encapsulated in PLGA microspheres was placed in a glass vial and 800 μl of phosphate buffered saline (50 mM, pH 7.4) was added. The vials were kept in a shaking water bath at 37°C. After specified time intervals, the reservoir buffer was collected by centrifugation at 5,000 g for 5 min, fresh buffer was added to the vials and kept in a water bath for further release studies. Blank microspheres were treated in a similar manner and served as control throughout the period of the experiment. The supernatant obtained was analyzed for protein content by bicinchoninic acid protein estimation method (30) and for antigenicity determination by ELISA (14).

## RESULTS

### Selection of Tetanus Toxoid-Specific RNA Libraries

The presence of $Mg^{2+}$ ions assists the folding of RNA into compact structures (31). Variation in the concentration of $Mg^{2+}$ ions can result in RNA structures with differing degrees of compactness. Since the recognition of the target by the RNA aptamer is based upon shape complementarity (16,32,33), the presence of different structures increases the diversity of the RNA pools further. For selection of tetanus toxoid-specific binder sequences, the toxoid ($M_r$ ~150 kDa) was immobilized on cyanogen-bromide activated agarose resin (6.6 nmol $ml^{-1}$). The RNA pools, T1 and T2, were incubated separately with immobilized tetanus toxoid in the presence of different concentrations of $Mg^{2+}$ ions in the selection buffer (2 mM, 3 mM and 4 mM). After ten cycles, dot blot assay was carried out to determine the affinity of the enriched RNA pools for tetanus toxoid. Both T1 and T2 libraries showed high affinity for the enriched RNA pools obtained after ten rounds of selection (Fig. 1). The starting RNA libraries had negligible affinity for tetanus toxoid, showing that the successive rounds of selection had enriched the RNA libraries for tetanus toxoid-specific sequences. The enriched libraries also showed negligible affinity for alcohol dehydrogenase ($M_r$ ~149.5 kDa) (data not shown), confirming specific recognition of their target. The enriched RNA library T1, selected at 2 mM $MgCl_2$, labelled as 2T1, exhibited a dissociation constant of 217±17 nM for tetanus toxoid. Enrichment of 2T2, 3T2 and 4T2 libraries showed corresponding $K_d$ values of 442±23, 150±16 and 240±20 nM for tetanus toxoid (Fig. 1). The affinity of the initial randomized library for the target protein was also determined by the same method. In this case, however, the concentration of the target protein was weighted towards the higher end to eliminate any kind of affinity for it whatsoever. Negligible binding of the randomized zero pool library with tetanus toxoid was observed, which confirmed the specificity of interaction of the enriched libraries for the target protein. 3T1 and 4T1 enriched libraries showed high values of dissociation constants and were not used further.

### Specific RNA Aptamers Inhibit Moisture-Induced Aggregation of Tetanus Toxoid

We have earlier reported the optimization of accelerated storage conditions for tetanus toxoid and have shown that exposure to 86% RH (provided by a saturated solution of $K_2CrO_4$) for 3 days resulted in an aggregated protein structure which best resembled the structure of the toxoid formed inside control release devices (10). Longer period of incubation (10 days) did not result in any increase in the fraction of aggregated protein (data not shown). Along with being a model for exposure of solid tetanus toxoid to elevated humidity level, the condition also reproduces the fate of tetanus toxoid inside such devices (10). We have employed this condition to monitor the effect of aptamers in increasing the stability of tetanus toxoid upon exposure to moisture *in vitro*.

In the primary screening step, the ability of monoclonal RNA sequences to alter the extent of moisture-induced aggregation of tetanus toxoid was monitored (Figure S1). The amount of soluble protein present in the supernatant after incubation of tetanus toxoid with monoclonal RNA sequences was measured. The list of clones which yielded the RNA sequences used for various studies is provided in Table I. Two RNA sequences from the enriched 2T1 library, TT-2 and TT-3, showed significant decrease in aggregation of tetanus toxoid upon exposure to 86% RH (Table I) as compared to control (84% aggregation). Four RNA sequences from the enriched 2T2 library, TT-4, TT-5, TT-6 and TT-7, showed substantial reduction in aggregation of the toxoid upon exposure to moisture (Table I). Eleven monoclonal RNA sequences from 3T2 library showed marked decrease in aggregation of tetanus toxoid upon

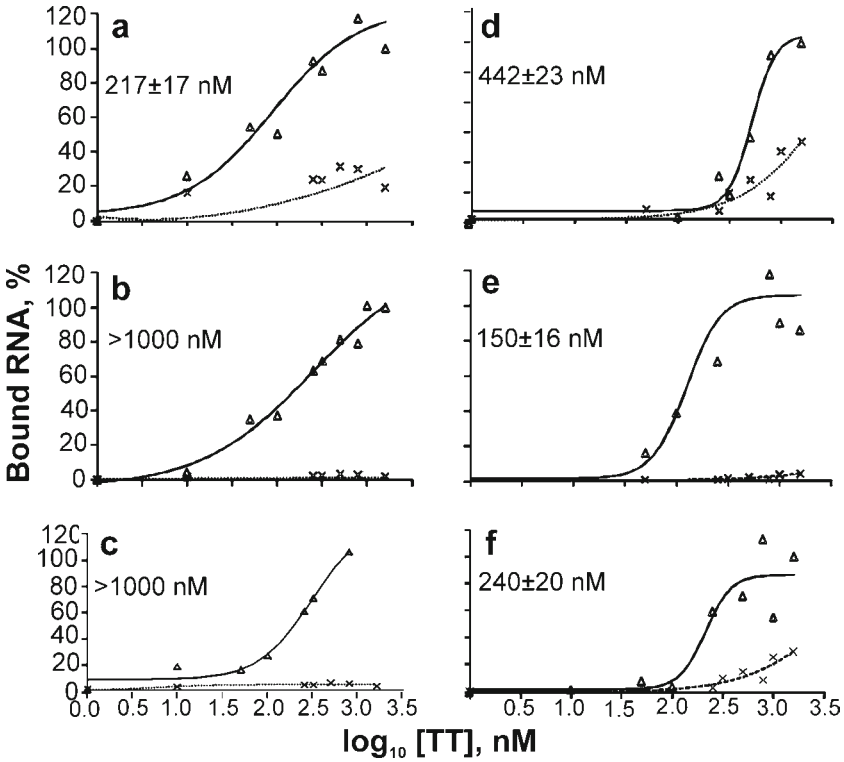


**Fig. 1** Estimation of dissociation constants ($K_d$) for the enriched RNA libraries by dot blot assay. The fluorescence intensity of the retained tetanus toxoid (TT)-RNA complex was quantified and fitted into the equation for a sigmoidal curve. The values written on the graphs indicate $K_d$ values for (**a**) enriched T1 selected at 2 mM $MgCl_2$ (2T1), (**b**) enriched T1 selected at 3 mM $MgCl_2$ (3T1), (**c**) enriched T1 selected at 4 mM $MgCl_2$ (4T1), (**d**) enriched T2 selected at 2 mM $MgCl_2$ (2T2), (**e**) enriched T2 selected at 3 mM $MgCl_2$ (3T2) and (**f**) enriched T2 selected at 4 mM $MgCl_2$ (4T2). The estimation was done for tenth cycle RNA pool (△, *solid line*) and the corresponding initial RNA library (X, *dashed line*). $K_d$ values shown on the graphs represent mean ± s.e.m. for three independent measurements.

exposure to 86% RH (Table I). Eight RNA sequences from the enriched 4T2 library showed considerable decrease in aggregation of tetanus toxoid on exposure to an environment of a saturated solution of $K_2CrO_4$ (Table I). Monoclonal RNA sequences from 3T2 and 4T2 libraries showed a higher reduction in moisture-induced aggregation of tetanus toxoid than sequences from 2T1 and 2T2 libraries. Since the dissociation constants of all these enriched libraries for tetanus toxoid lie in a similar range (Fig. 1), members of these four libraries bind to tetanus toxoid with high affinity. The successful sequences from 3T2 and 4T2 libraries possibly bind to regions of tetanus toxoid which are important in the initiation of moisture-induced aggregation of the protein and thus act as inhibitors of aggregation. This has been investigated further later on.

In the secondary screening step, a dual-parametric strategy was employed. Measurement of residual antigenicity was taken as a second criterion along with the extent of aggregation of the toxoid in the presence of monoclonal RNA sequences. Aggregation of the toxoid exposed to moisture was measured again using those RNA sequences whose presence resulted in reduction in protein aggregation in the primary screening step. Significant decrease in the aggregation of tetanus toxoid in the presence of the RNA sequences (Fig. 2a) confirmed the success of this approach.

In order to determine whether decrease in aggregation of the protein correlated with retention of its antigenicity, ELISA of tetanus toxoid subjected to moisture-induced aggregation in the presence of RNA aptamers was carried out using mouse anti-tetanus toxoid monoclonal antibody as the primary antibody (14). All the sequences which prevented aggregation of tetanus toxoid also showed significant retention of antigenicity of tetanus toxoid albeit up to different extents (Fig. 2b). The control sample, incubated in the absence of any RNA sequence, showed residual antigenicity of 26% upon exposure to moisture. In the presence of RNA sequences like TT-13, TT-17, TT-20, TT-22, TT-23, TT-24, TT-25, TT-26, TT-27 and TT-29, tetanus toxoid could retain more than 60% antigenicity upon exposure to moisture, which reflected >2.3-fold improvement in retention of antigenicity. The antigenicity of tetanus toxoid was also determined in the RNA-bound form (without exposure to moisture). In none of the cases was the antigenicity found to be significantly different from that of the unbound or native tetanus toxoid (Figure S2). Thus, the binding of the RNA aptamer to tetanus toxoid does not affect the binding of the monoclonal antibody to it. The epitope recognized by the mouse monoclonal antibody is not defined by the manufacturer. Antigenicity estimation of the protein-RNA complex, after exposure to moisture, was repeated by ELISA using human polyclonal tetanus toxoid anti-serum. Similar values of antigenicity were obtained with monoclonal and polyclonal antibodies (Fig. 2b), indicating the reliability of the results. During unfolding and aggregation of tetanus toxoid, the epitopes recognized by the monoclonal and polyclonal antibodies may be denatured. Hence, aggregation of the protein is closely correlated with its loss of conformation, measured as reduction of antigenicity.

**Table I** List of RNA Sequences from Enriched Libraries Used for Various Studies in this Work. 100% Aggregation Assumes that All of the Initially Added Toxoid has Aggregated

| Label | Library | Concentration of $Mg^{2+}$ used for selection | Clone number | Aggregation,% |
|---|---|---|---|---|
| TT-1 | T1 | 2 mM | 5 | 82 |
| TT-2 | T1 | 2 mM | 32 | 59 |
| TT-3 | T1 | 2 mM | 33 | 54 |
| TT-4 | T2 | 2 mM | 14 | 59 |
| TT-5 | T2 | 2 mM | 16 | 58 |
| TT-6 | T2 | 2 mM | 28 | 54 |
| TT-7 | T2 | 2 mM | 30 | 60 |
| TT-8 | T2 | 2 mM | 31 | 67 |
| TT-9 | T2 | 3 mM | 21 | 52 |
| TT-10 | T2 | 3 mM | 25 | 33 |
| TT-11 | T2 | 3 mM | 27 | 37 |
| TT-12 | T2 | 3 mM | 30 | 48 |
| TT-13 | T2 | 3 mM | 34 | 33 |
| TT-14 | T2 | 3 mM | 36 | 86 |
| TT-15 | T2 | 3 mM | 42 | 36 |
| TT-16 | T2 | 3 mM | 43 | 45 |
| TT-17 | T2 | 3 mM | 44 | 38 |
| TT-18 | T2 | 3 mM | 45 | 37 |
| TT-19 | T2 | 3 mM | 48 | 49 |
| TT-20 | T2 | 3 mM | 49 | 41 |
| TT-21 | T2 | 4 mM | 2 | 83 |
| TT-22 | T2 | 4 mM | 11 | 44 |
| TT-23 | T2 | 4 mM | 13 | 48 |
| TT-24 | T2 | 4 mM | 14 | 38 |
| TT-25 | T2 | 4 mM | 15 | 44 |
| TT-26 | T2 | 4 mM | 18 | 40 |
| TT-27 | T2 | 4 mM | 25 | 43 |
| TT-28 | T2 | 4 mM | 28 | 47 |
| TT-29 | T2 | 4 mM | 50 | 45 |

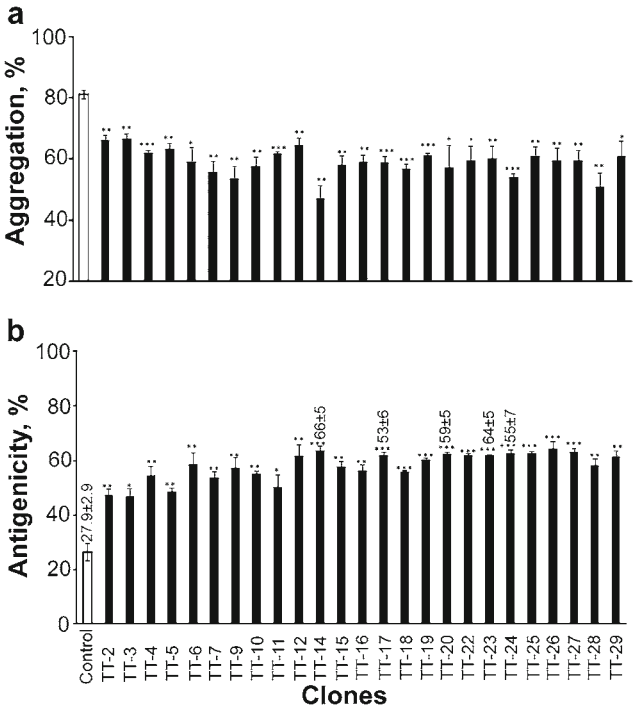


**Fig. 2** Effect of RNA sequences on the (**a**) aggregation and (**b**) antigenicity of tetanus toxoid exposed to moisture. Moisture-induced aggregation of tetanus toxoid was carried out in the presence of RNA sequences obtained from enriched pools and are denoted as *filled bars*. Tetanus toxoid was also incubated under test conditions in the absence of any RNA sequence (*empty bar*). 100% aggregation assumes that all of the initially added protein has aggregated whereas 100% antigenicity refers to the antigenicity of native tetanus toxoid prior to exposure to moisture. *Bars* in Fig. 2b indicate antigenicity values obtained using anti-tetanus toxoid monoclonal antibody as the primary antibody. Values above the bars show% residual antigenicity values obtained in the presence of arbitrary RNA aptamer sequences by ELISA using human polyclonal tetanus toxoid anti-serum. *** represents $p < 0.001$ *vs.* control, ** represents $p < 0.01$ *vs.* control, * represents $p < 0.05$ *vs.* control. p-values were calculated using Student's *t*-test. All experiments were carried out in triplicate and mean ± s.e.m. values are shown.

## Analysis of Tetanus-Toxoid-Specific RNA Aptamers

The affinity of the RNA aptamers for tetanus toxoid was determined by dot-blot assay. Data obtained were fitted to a sigmoidal curve and dissociation constants were calculated (25) (Table II). All the aptamers showed high affinity for the toxoid, as indicated by dissociation constants in nanomolar range. Dissociation constants were also estimated for some other sequences from the enriched libraries which did not inhibit aggregation of the toxoid, *viz.* TT-1, TT-8, TT-14 and TT-21. These sequences were also shown to have similar affinity for tetanus toxoid as those which inhibited moisture-induced aggregation of the latter (Table II). Thus, the assumption of the earlier section, that there are two classes of binders, *viz.* inhibitors and non-inhibitors, with similar affinities for tetanus toxoid but differing in their binding sites on the protein, is plausible.

Individual inhibitor aptamers were cloned and sequenced. The sequences were analyzed by multiple alignment using T-COFFEE (tree-based consistency objective function for alignment evaluation) web server (34) to determine the pattern of different sequences for the identification of consensus regions. All the sequences showed conserved regions at the ends of the variable expanse (Table III). Apart from this, no region of significantly shared sequence could be found. We also attempted to align the sequences using the frequently used tool CLUSTALW (35) (data not shown). However, we did not find any region of significant similarity. The lower accuracy of CLUSTALW as compared to the newer tools like T-COFFEE, PROBCONS, MAFFT, *etc.* has been reported by experts working in the field (36). Proteins with high molecular weights have been reported

**Table II** Affinity of Monoclonal RNA Sequences for Tetanus Toxoid. The Ability of Monoclonal RNA Sequences to Inhibit Moisture-Induced Aggregation of Tetanus Toxoid has been Described in Fig. 3. Dissociation Constants for Sequences Which Act as Inhibitors and which Do Not Inhibit Aggregation of Tetanus Toxoid were Determined by Labeling RNA Sequences with Fluorescein. Values Represent Mean ± s.e.m. of Three Independent Experiments

| Monoclone | Dissociation constant ($K_d$, nM) | Binder | Inhibitor |
|---|---|---|---|
| TT-9 | 206 ± 53 | ✓ | ✓ |
| TT-12 | 101 ± 9 | ✓ | ✓ |
| TT-13 | 142 ± 22 | ✓ | ✓ |
| TT-17 | 271 ± 11 | ✓ | ✓ |
| TT-20 | 224 ± 25 | ✓ | ✓ |
| TT-23 | 223 ± 22 | ✓ | ✓ |
| TT-24 | 181 ± 40 | ✓ | ✓ |
| TT-25 | 122 ± 7 | ✓ | ✓ |
| TT-26 | 182 ± 42 | ✓ | ✓ |
| TT-27 | 135 ± 15 | ✓ | ✓ |
| TT-28 | 210 ± 25 | ✓ | ✓ |
| TT-29 | 265 ± 17 | ✓ | ✓ |
| TT-1 | 127 ± 25 | ✓ | × |
| TT-8 | 423 ± 61 | ✓ | × |
| TT-14 | 198 ± 12 | ✓ | × |
| TT-21 | 217 ± 33 | ✓ | × |

to have tight binding aptamer sequences with no consensus regions between them. For example, six RNA aptamers selected against the oligomeric extracellular domain of the human epidermal growth factor receptor-3 (ECDHER3, $M_r$ ~ 980 kDa of ECD oligomer) failed to show any consensus sequence (37). The authors speculated that there could be multiple binding sites on the very large protein, which gave rise to unique RNA sequences.

The local similarity between different structures was analyzed using SimTree web based server (http://bioinfo.cs.technion.ac.il/SimTree/). For this, similarity scores between two secondary structures were calculated and normalized for the sizes of different sequences (Table IV). A normalized score of 0 reflects no match whereas 1 indicates a perfect match. Values from 0 to 1 indicate increasing match. Different sequences showed varying degrees of similarity. Comparison between inhibitors showed similar normalized scores in the range of 0.7191–0.7993. Comparison of inhibitors with binders alone showed significantly higher variation (0.5612–0.8719). There is no significant similarity, either at the primary sequence or at the secondary structure level, between inhibitor sequences. However, the secondary structures of the inhibitors are significantly different from the non-inhibitors (data not shown). Aptamers bind to their targets on the basis of shape complementarity (16). This difference in shape may account for the inability of the latter sequences to inhibit moisture-induced aggregation of tetanus toxoid, which could account for the higher variation in the normalization scores.

## Encapsulation of Tetanus Toxoid in PLGA Microspheres

Tetanus toxoid-RNA complexes were encapsulated in PLGA microspheres. The surface morphology of microspheres was analyzed by scanning electron microscopy. Micrographs showed microspheres with smooth surfaces in both cases (data not shown). The majority of particles were observed to be in the size range of 2–5 μm which matched

**Table III** Multiple Sequence Alignment of Sequences. Sequences were Aligned Using T-Coffee Web Server (34) for Multiple Sequences Alignment

| Sequence ID | Alignment | |
|---|---|---|
| TT-1 | GACAC————GCG—GC————T–CG-T-CTGGCC | 20 |
| TT-8 | CCCTGGGACTAGGGGCAGGCTAATAACCCA-A-CTGGCC | 37 |
| TT-9 | CGGATAGGT–GCA—GTAGAGCAA-AAC-A-CCGCCC | 31 |
| TT-12 | AGACGAAGCAAGATATAGGGCAATAACCCG-C-GGTCCC | 37 |
| TT-13 | GAGCGCGAT-ATCGTAAGCAAAAGCT-CTCTAGCGGGCC | 37 |
| TT-14 | CTTTGGAGACAGCGGCTGCCCTACTGAGCC-C-TACCCC | 37 |
| TT-17 | CTCATAAAA-ACC–AAGGGAAATGCCCAGCC-CCCCCC | 35 |
| TT-20 | GGGTCCCCA-AGGGTAACCAAAGAGAAACCTC-GTCCGC | 37 |
| TT-21 | CGAAGAGACCATCAA-CGAGGATC-CAAAAGG-CCCGGC | 36 |
| TT-23 | CTCGGAGATTATCGG-ATAAGAGCATGGTCTA-CCCGGC | 37 |
| TT-25 | CAAAC-GATAATC-GAAGCAAGAAACGCCATC-ATCGCC | 36 |
| TT-26 | GCAGGAAACAGTCCA-GTACTTTAGTTACCCA-TCACCC | 37 |
| TT-27 | CGGATGTCG-GCAGTCATAATAGTAACTCGTT-CGTGCC | 37 |
| TT-28 | GCCATGGCA-ACATAGGGTAAAATACGTCCGA-ACCACC | 37 |
| TT-29 | TTCATGACAAACAGGAG-GGGGAGATTTCACG-TCCGCC | 37 |
| * | | |

**Table IV** Comparison of Local Similarity Between Various Sequences Normalized Scores for Various Sequences

| Inhibitor | Inhibitor | Normalized score |
|---|---|---|
| Comparison between sequences which bind and inhibit moisture-induced aggregation of tetanus toxoid | | |
| TT-13 | TT-17 | 0.7625 |
| | TT-20 | 0.7935 |
| | TT-23 | 0.7993 |
| TT-17 | TT-20 | 0.7844 |
| | TT-23 | 0.7347 |
| TT-20 | TT-23 | 0.7191 |
| Comparison between sequences which inhibit moisture-induced aggregation of tetanus toxoid with those sequences which are non-inhibitors | | |
| Inhibitor | Non-Inhibitor | Normalized score |
| TT-13 | TT-1 | 0.7276 |
| | TT-8 | 0.6638 |
| | TT-14 | 0.6737 |
| | TT-21 | 0.7450 |
| TT-17 | TT-1 | 0.5612 |
| | TT-8 | 0.8463 |
| | TT-14 | 0.8705 |
| | TT-21 | 0.7313 |
| TT-20 | TT-1 | 0.7226 |
| | TT-8 | 0.8526 |
| | TT-14 | 0.8719 |
| | TT-21 | 0.7201 |
| TT-23 | TT-1 | 0.7022 |
| | TT-8 | 0.6295 |
| | TT-14 | 0.6458 |
| | TT-21 | 0.8027 |

well with what is reported in the literature (30, 33). Zeta potential of the microspheres was estimated to determine the surface charge on them. This parameter is an indicator of the stability of colloidal systems. A high value of zeta potential indicates sufficient repulsion between the charged surface groups, which keeps the system stable. Negative zeta potential of magnitude more than -20 mV was observed in all cases (Table V). This indicated the stability of prepared microspheres in suspension.

## Release Profile of Tetanus Toxoid from PLGA Microspheres

*In vitro* release profile of tetanus toxoid from microspheres was monitored over a period of 55 days. All the preparations showed a burst release in the first 2 h followed by continuous release of the toxoid (Fig. 3a). Control sample, which

**Table V** Measurement of Zeta Potential of PLGA Microspheres Containing Tetanus Toxoid-RNA Complexes. Values Represent Mean ± s.e.m. of Three Independent Experiments

| Sample | Zeta potential (mV) |
|---|---|
| Microspheres containing | |
| Tetanus Toxoid alone | −23.5 ± 1.4 |
| Tetanus Toxoid + TT-13 | −25.8 ± 2.7 |
| Tetanus Toxoid + TT-17 | −25.3 ± 0.6 |
| Tetanus Toxoid + TT-20 | −23.4 ± 1.3 |
| Tetanus Toxoid + TT-23 | −25.6 ± 3.2 |
| Tetanus Toxoid + TT-24 | −27.8 ± 1.0 |
| Non encapsulated | |
| Tetanus Toxoid alone | −12.3 ± 1.6 |
| Tetanus toxoid + TT-13 | −20.9 ± 0.5*** |
| Tetanus toxoid + TT-23 | −16.4 ± 1.7* |

*$p < 0.05$, ***$p < 0.001$ against tetanus toxoid alone

represents the release of tetanus toxoid from microspheres in the absence of RNA, showed release of 26 ± 1% release of total encapsulated protein after a period of 55 days. The aggregation of the toxoid inside the microspheres has been postulated to be one of the major reasons for the incomplete release of protein (14). Tetanus toxoid incubated with RNA sequences TT-13, TT-17, TT-20, TT-23 and TT-24 showed release of 54 ± 4%, 47 ± 4%, 40 ± 4%, 55 ± 4% and 41 ± 5% protein, respectively, at the end of the study period. The release of a higher amount of toxoid in the presence of RNA sequences as compared to the control microspheres (no RNA) indicated that the RNA aptamers prevented the aggregation of tetanus toxoid encapsulated inside the microspheres and thus enhanced its release.

Antigenicity of the released protein was analyzed by ELISA (14) using mouse anti tetanus toxoid monoclonal antibody as the primary antibody. The cumulative antigenicity of the released protein was plotted against time (Fig. 3b). Significantly higher antigenic protein was released from the microspheres encapsulating tetanus toxoid-RNA complex as compared to those of control (protein alone), reflecting the retention of the epitope of the protein in the former case. The control microspheres, containing only tetanus toxoid, showed 11 ± 1% antigenicity of the released protein. Tetanus toxoid incubated with RNA sequences TT-13, TT-17, TT-20, TT-23 and TT-24 showed 46 ± 1%, 34 ± 3%, 25 ± 5%, 44 ± 3% and 35 ± 2% antigenicity of the released protein, respectively. This indicated that the RNA sequences reduced aggregation and preserved the antigenically-active conformation of tetanus toxoid inside the microsphere. Since RNA degrades under the conditions used to dissolve microspheres and extract the encapsulated complex, we could not determine the actual amount of RNA present in the microspheres. However, the significant increase in sustained release of tetanus toxoid in case of

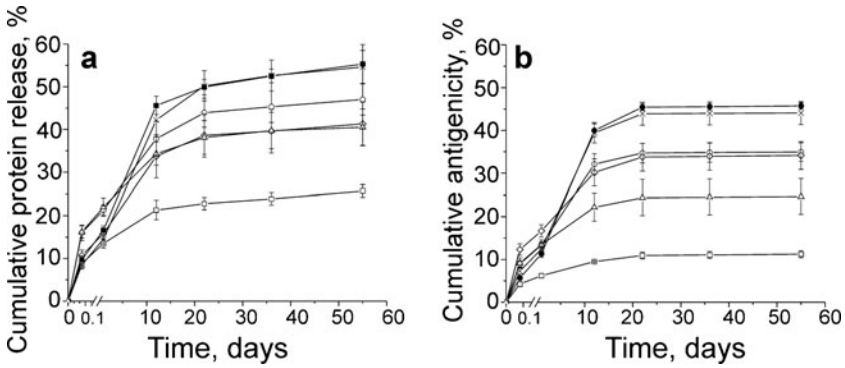


**Fig. 3** *In vitro* release profile of tetanus toxoid from PLGA microspheres. The amount (**a**) and antigenicity (**b**) of released protein were measured. Release studies were performed for microspheres containing tetanus toxoid only (□) and complexed with TT-13 (X), TT-17 (○), TT-20 (△), TT-23 (■) and TT-24 (◇). 100% release assumes that all of the encapsulated protein has been released and 100% antigenicity corresponds to antigenicity of the total toxoid encapsulated initially. Values represent mean ± s.e.m. of three independent experiments.

encapsulation with RNA confirmed the presence of RNA within the microspheres.

## Mechanism of Stabilization of Tetanus Toxoid by RNA Aptamers Inside PLGA Microspheres

Surface morphology of the microspheres was observed by scanning electron microscopy after the completion of release studies and compared to that of starting microspheres (data not shown). Initially, the appearance of the surface of microspheres encapsulating tetanus toxoid or tetanus toxoid-RNA complex was smooth and did not show any crevices. After the completion of the study period (*i.e.* 55 days), PLGA microspheres, encapsulating either tetanus toxoid alone or complexed with RNA sequences, showed wrinkled topology. Thus, the release of more amount of antigenically active toxoid over a longer period of time was not because of any effect of RNA sequences on the PLGA microspheres against degradation which might have protected the toxoid against moisture-induced aggregation.

The specific antigenicity of the released toxoid, *i.e.* the antigenicity of the toxoid per μg of protein, was calculated and plotted against time (Fig. 4). All the preparations showed decrease in specific antigenicity with time. The loss was faster in case of the control tetanus toxoid sample incubated in the absence of any RNA sequence. This indicated that in the initial stages of release (~20 days), the RNA sequences facilitated the retention of antigenic conformation of tetanus toxoid. With time, the release of antigenically active protein slowed down (Fig. 4), so that the overall antigenicity of the released fraction reached a plateau. The rate of decrease of specific antigenicity of the toxoid released from the microspheres in the presence of RNA aptamers was lower as compared to that of control (without RNA) (Fig. 4). Thus, aptamer-bound tetanus toxoid remained antigenically active inside the microspheres, which facilitated its continual release.

Till date, no antigenicity or toxicity has been associated with the use of aptamers (18–20). Since the affinity of the RNA aptamers for tetanus toxoid is high (Table II), it is probable that the protein is released from the microspheres in the RNA-bound form. However, no RNA could be detected in the release medium. In order to determine the fate of RNA even it is released into the circulatory system after the administration of the microspheres, we exposed the RNA sequence to serum (diluted fifty times) in a separate experiment, to mimic such a situation. We were not able to detect any RNA sequence by reverse transcription-polymerase chain reaction (RT-PCR) after incubation with serum for 15 min (data not shown). This confirmed that upon administration of the microspheres in the body, unmodified RNA, even if released, will be inactivated immediately by circulatory nucleases.

There are two possible routes by which RNA aptamers can stabilize tetanus toxoid. The RNA sequence could be bound to the surface of the toxoid more prone to absorbing moisture and undergoing unfolding, which would be the first step of aggregation of the protein; the RNA aptamer might inhibit the absorption of moisture by the protein.

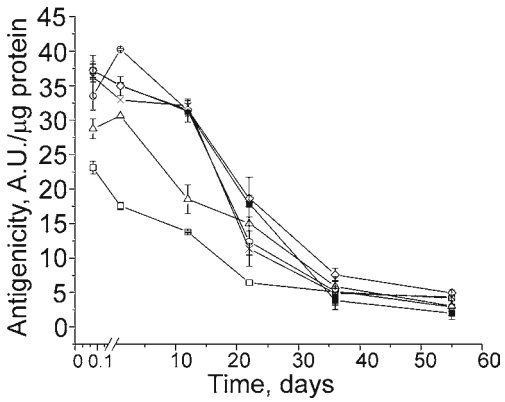


**Fig. 4** Specific antigenicity of released tetanus toxoid from PLGA microspheres. Rest of the legend is similar to Fig. 3.

Conversely, the presence of the charged RNA molecule could help in absorbing more moisture (water), thus solubilising the protein. The amount of water absorbed by the lyophilized protein and the lyophilized protein-RNA complexes incubated under 86% RH (provided by a saturated solution of $K_2CrO_4$) was determined by Karl Fischer titration. No significant difference in the amount of water absorbed by the protein in the absence and presence of RNA sequences could be determined (106±5 and 104±4 g water/100 g protein for tetanus toxoid complexed with TT-13 and TT-23, respectively, against 97±4 g water/100 g protein for control toxoid without RNA). Thus, the decrease in aggregation of tetanus toxoid observed in the presence of RNA was not because of any change in the amount of water absorbed in the latter case.

## DISCUSSION

Recently, anti-platelet-derived growth factor-BB (PDGF-BB) aptamer has been used for sustained release of PDGF-BB from polyacrylamide gel and poloxamer hydrogel. The aptamers, exhibiting high affinity for the protein, were immobilized on matrices, and were successful in slowing down the release of the protein from the gel matrix (38, 39). The release profile, however, was monitored only for 196 h. In this and other cases, the selected aptamer was used to crosslink the polymer and the release of the protein was slowed down because the protein was (transiently) immobilized inside the microsphere (40, 41). The stabilization of the protein inside the microspheres, if any, was not described.

It has been reported that in the presence of certain excipients, the amount of water absorbed by the solid protein increases and hence, the protein exists in the soluble form. In the presence of RNA sequences, the amount of water absorbed by the solid toxoid was found to be similar to the amount of water absorbed by the solid toxoid alone. Thus, the presence of RNA aptamers inhibits unfolding of the toxoid during exposure to moisture. This is likely to be the cause for the stabilizing effect of RNA aptamers observed here (Fig. 5). During incubation of the protein under elevated moisture conditions, the structural flexibility of the protein increases due to absorption of water, leading to the exposure of the buried hydrophobic core of the native conformation. This acts as the starting point of protein aggregation. The presence of specific RNA aptamers would inhibit this process. This hypothesis also agrees well with the results observed. If RNA aptamers were simply facilitating absorption of more water, stabilization would be observed with all sequences irrespective of their secondary structures. This is not seen in the present case. Instead, specific sequences bind to tetanus toxoid and inhibit its unfolding, which is the first step of denaturation and/or aggregation.

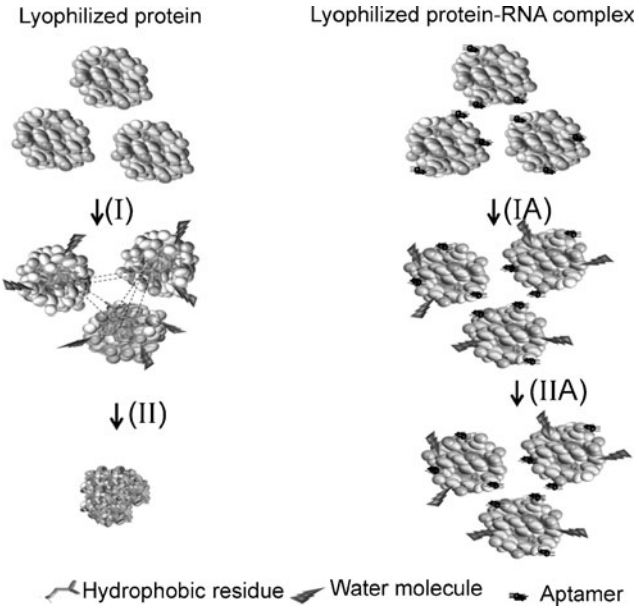


**Fig. 5** Probable mechanism of stabilization of tetanus toxoid inside PLGA microspheres. On exposure to moisture, (I) the lyophilized protein absorbs water molecules, becomes flexible and is partially unfolded. Exposure of the buried hydrophobic core in neighbouring protein molecules leads to interaction among them and ultimately, (II) formation of aggregates, which are functionally inactive. In the presence of RNA aptamers, (IA) the unfolding of the protein in the presence of water is hindered sterically. Since the hydrophobic residues are not exposed to the aqueous environment, interaction between protein molecules is not possible. In addition, protein molecules remain in solution due to repulsion between like charges on the protein molecules in the presence of RNA sequences. (IIA) Since unfolding does not occur and the protein molecules cannot interact with each other, aggregation is reduced and tetanus toxoid retains its antigenicity.

RNA has negative charge and in its protein-bound form, imparts negative charge to the native conformations of proteins (42–44). The electrostatic repulsion between negatively charged protein-RNA complexes probably keeps protein molecules apart under elevated moisture conditions. In the present case, a significant increase in the surface charge of the RNA-protein complex could be observed as compared to the charge on the protein alone (Table V). This prevents interaction between protein molecules and is an additional factor in reducing the aggregation of the protein. The mechanism by which aptamers stabilize proteins is, thus, different from small molecules.

Stabilization of tetanus toxoid against moisture-induced aggregation using small molecules has been reported by other groups. For instance, the use of sorbitol resulted in ~80% soluble protein at the end of the stress period (45). Similarly, stabilization studies of model formalinized antigens with L-lysine have yielded ~100% soluble protein at the end of the incubation period, compared to 11% in the absence of any stabilizer (9). No functional data, *e.g.* antigenicity or activity, was presented in either case. Significant stabilization had also been reported in the case of encapsulated tetanus

toxoid against aggregation in the presence of a combination of three stabilizers (15). In another report, tetanus toxoid was encapsulated in PLGA microspheres and stabilized with the disaccharide trehalose, a commonly used protein stabilizer (28). About two-fold improvement in antigenic integrity of the released toxoid was reported.

In this work, we have proposed a novel approach for stabilization of proteins; one which depends on interaction of the stabilizer with the protein rather than the solvent. In addition to its role as a stabilizer of tetanus toxoid when exposed to moisture, it is possible that the aptamers inhibit the adsorption of the protein to either the oil–water interface or the matrix (PLGA) and thus, protect the toxoid during processing stress. However, no difference in specific antigenicity of tetanus toxoid could be detected after exposure to the oil–water interface in the absence or presence of the RNA aptamer, TT-13 ($p > 0.05$, data not shown). It has been reported that polyols such as trehalose and glycerol were able to stabilize a monoclonal antibody against thermal stress (measured as an increase in melting temperature, $T_m$) and decrease its aggregation (46). However, no protective role was seen when the protein solution was agitated at 200 rpm for 5 days (mechanical stress). We have also recently observed that although trehalose was able to retain the antigenicity of an aluminium hydroxide adjuvant preparation of tetanus toxoid against freeze-thawing (47), the disaccharide was unable to maintain the integrity of the adjuvanted formulation against agitation-induced stress (48). Hence, the stabilization offered by osmolytes is *against* the stress condition but not particularly *for* the protein molecule. Nucleic acid aptamers, on the other hand, recognize their targets with high affinity and specificity. The mechanism by which they inhibit protein-protein interaction, which is the first step in protein aggregation or protein unfolding, is by specifically interacting with their targets and sterically blocking specific regions on the protein molecule.

## CONCLUSION

Aggregation of tetanus toxoid inside PLGA microspheres has been identified as one of the main reasons for incomplete release of protein and failure of single-dose vaccines. In the present work, we have studied the effect of these RNA aptamers on the aggregation of tetanus toxoid encapsulated in PLGA microspheres and elucidated the mechanism of stabilization of the protein. Sustained release of the antigen could be observed up to 55 days of study. The interaction of the stabilizer (aptamer) with the protein and not the solvent is different from that of conventional small molecule osmolytes and is likely to widen the range of stress conditions against which the aptamer can be successfully employed. It is anticipated that aptamers will be able to act as universal inhibitors of target proteins and inhibit the unfolding and denaturation of the protein against different kinds of stress factors. We have initiated experiments to test this hypothesis.

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