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RESEARCH ARTICLE

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Coupling enterotoxigenic *Escherichia coli* heat-stable peptide toxin with 8-arm PEG enhances immunogenicity

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Enterotoxigenic *Escherichia coli* (ETEC) strains, which produce the heat-stable enterotoxin (ST) either alone or in combination with the heat-labile enterotoxin, contribute to the bulk of the burden of child diarrheal disease in resource-limited countries and are associated with mortality. Developing an effective vaccine targeting ST presents challenges due to its potent enterotoxicity, non-immunogenicity, and the risk of autoimmune reaction stemming from its structural similarity to the human endogenous ligands, guanylin, and uroguanylin. This study aimed to assess a novel synthetic vaccine carrier platform employing a single chemical coupling step for making human ST (STh) immunogenic. Specifically, the method involved cross-linking STh to an 8-arm N-hydroxysuccinimide (NHS) ester-activated PEG cross-linker. A conjugate of STh with 8-arm structure was prepared, and its formation was confirmed through immunoblotting analysis. The impact of conjugation on STh epitopes was assessed using ELISAs with polyclonal and monoclonal antibodies targeting various epitopes of STh. Immunization of mice with the conjugate induced the production of anti-STh antibodies, exhibiting neutralizing activity against STh.

KEYWORDS
bioconjugation, diarrhea, enterotoxigenic *Escherichia coli* (ETEC), ETEC vaccine, heat-stable enterotoxin (ST), immunogenicity, multi-arm PEG cross-linking

1 | INTRODUCTION

Enterotoxigenic *E. coli* (ETEC) is a major cause of diarrheal disease in children under 5 years of age in resource-limited countries. Particularly, ETEC strains that produce the heat-stable enterotoxin (ST) with or without the heat-labile enterotoxin contribute to the bulk of the burden of child diarrheal disease in these countries and are associated with mortality.[1–3] ST peptides function as super agonists for the transmembrane guanylyl cyclase-C (GC-C) receptors, which are primarily found in the small intestine.[4] The GC-C receptors are typically regulated by the endogenous peptides guanylin and uroguanylin; however, ST exhibits a significantly higher affinity for binding to this receptor.[4,5] Upon ST binding to the GC-C receptor, its intracellular cyclase domain catalyzes the conversion of GMP to cGMP, initiating a signaling cascade. This cascade leads to the secretion of Cl^- and HCO^{3-} ions and inhibits Na^+ absorption via the sodium–hydrogen exchanger.[6,7] These cascades ultimately result in a watery diarrhea.

To date, no licensed vaccine for ETEC exists. However, efforts are currently underway to develop a vaccine against ETEC. Among the leading ones are ACE527, a live attenuated ETEC vaccine candidate[8] and an oral inactivated vaccine, ETVAX®.[9] ST-based vaccine efforts are also underway.[10,11] The development of an ST-based vaccine has been challenging due to several factors: ST's potent enterotoxicity, lack of immunogenicity, and its similarity to human hormones

guanylin and uroguanylin, which may trigger an autoimmune reaction.[12–14] To overcome the enterotoxicity and autoimmune risks, specific mutations in ST need to be made while preserving essential epitopes that generate neutralizing antibodies.[10,13,15] In this regard, promising single and double ST mutants that eliminate toxicity and minimize the risk of cross-reaction with endogenous ligands have been identified.[10,11,15,16]

ST, variants STh (human) and STp (porcine), is a small peptide of approximately 2 kDa that does not inherently induce an immune response following natural ETEC infections. Conventional methods of eliciting an anti-ST immune response thus involve coupling ST to immunogenic proteins (carriers) through chemical cross-linking,[12] genetic fusion,[17,18] or using the spyTag-spyCatcher system.[10,19] The advantage of chemical conjugation compared to genetic fusion lies in the ability to thoroughly characterize ST peptides biochemically and biophysically to ensure intact protective epitopes before conjugation, as improperly folded ST toxoid may fail to present the necessary epitopes needed to stimulate the production of toxin-neutralizing antibodies.[14,20] Additionally, chemical conjugation allows for higher hapten-to-carrier ratios compared to genetic fusions and offers the possibility to produce toxoid candidates via chemical synthesis as well as recombinant methods. Nonetheless, both genetic fusion and chemical conjugation approaches have proven effective in stimulating antibody production against ST.[21–24] MecVax is indeed a genetic fusion multivalent ETEC vaccine candidate in the preclinical development stage, consisting of two units: an adhesin multiepitope fusion antigen (MEFA) that stimulates antibody production against the seven most important ETEC adhesins (CFA/I and CS1–CS6) and a toxoid fusion antigen that induces an antibody response against ETEC enterotoxins (heat-labile toxin and heat-stable toxin).[21]

Protein carriers offer the epitopes essential for recognition by T-helper cells in both genetic fusions and chemical conjugates, which allow for the generation of high-affinity antibodies and immune memory against peptide haptens.[25,26] Various proteins, including bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), heat-labile toxin subunit B (LTB), and viruslike particles (VLP) have been utilized as carrier proteins to make STh immunogenic.[10,14,19,27] Preexisting immunity against the commonly used protein carriers used in conjugate vaccines has been found to suppress the immune response to haptens, resulting in carrier-induced hapten-specific suppression.[28–30] This motivates the exploration of alternative vaccine carrier platforms for poorly immunogenic antigens such as ST.

In the past, homobifunctional cross-linkers like glutaraldehyde and carbodiimides were commonly employed to couple ST to carrier proteins[23,31–33] due to the relative simplicity of the conjugation process. However, these conjugation methods may lead to poorly defined conjugates, sometimes forming high molecular weight constructs that may precipitate.[34] Conversely, two-step conjugations using heterobifunctional cross-linkers offer a promising alternative, allowing for more controlled reactions and enabling the customization of peptide orientations on the carrier.[34] Despite the advantages of the latter approach, the elaborate and complex chemistry involved may increase the vaccine production cost,[35] highlighting the need for a simple chemistry that facilitates the production of well-defined conjugate vaccines.

In this study, we utilized 8-arm N-hydroxysuccinimide (NHS) ester-activated homofunctional cross-linker to couple eight STh peptides per cross-linker through the N-terminus of STh in a single step. Our results demonstrate that the 8-arm STh-PEG conjugate induced anti-STh antibodies in mice. Furthermore, the sera from immunized mice exhibit neutralizing activity against the native STh toxin.

2 | MATERIALS AND METHODS

2.1 | Recombinant STh peptide production

STh peptide (amino acid sequence: NSSNYCCELCCNPACTGCY) was overexpressed and purified using the DsbC-ST method previously described.[11] Briefly, pET-DsbC-STh plasmid was transformed into *E. coli* BL21 Star™ (DE3) (Invitrogen, Waltham, MA, USA), which were then cultured in 2YT medium supplemented with 2% (w/v) glucose and 50 μg/mL kanamycin. Induction was achieved using 0.5 mM IPTG. Following expression, cell lysis was carried out using lysozyme and ultrasonication. After removing cell debris via centrifugation, the cleared lysates underwent Ni-NTA purification. Subsequently, STh peptides were cleaved off from their DsbC fusion partner using Tobacco Etch Virus (TEV) protease. The fusion partner was eliminated through a second round of Ni-NTA purification, and the peptide-containing flow-through was subjected to reversed-phase chromatography. Fractions corresponding to distinct peaks were combined, and methanol was removed while concentrating the samples using a rotary evaporator (Rotavapor® R-100, Buchi, Flawil, Switzerland). The purified STh mutant peptide masses were confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) as described previously.[10] The peptide was dissolved in PBS buffer with the following composition: 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl, pH 7.2. Peptide concentrations in each of the pooled fraction samples were measured with a NanoDrop using the theoretical extinction coefficient of STh and the molecular weight in the calculations.

2.2 | Competitive ELISA

Following recombinant purification of STh, competitive ELISA was conducted as described previously[10,12] to identify the isomer of STh with the correct disulfide bridge connectivity. Briefly, Nunc Immobilizer amino microtiter plates (Thermo Scientific) were coated with 100 μL of PBS containing ~4 ng of STh per well and incubated overnight at 4°C. After blocking with 180 μL of 1% ovalbumin for 1 h with shaking, the plates were washed three times with PBST buffer (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl, pH 7.2, 0.05% Tween 20). Then, 60 μL of 1:8000 dilution of the C30 anti-STp monoclonal antibody (clone M120530 [Fitzgerald, North Acton, MA, UK]) and 60 μL of serial peptides dilutions of candidate STh isomers

were added. After a 1.5 h incubation, the wells were washed three times with PBST buffer and incubated with a 1:4000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) for 1 h. Following a final washing with PBST three times, an enzyme substrate was added, and absorbance was measured after a 20-min incubation at RT. The percentage inhibition of maximum binding was calculated as described previously.[10]

2.3 | Preparation of STh-8-arm conjugate

Initially, 20 mM stock solutions of 8-arm PEG-SC (Biopharma PEG, #A88023-10 K) cross-linker was prepared by dissolving in dry anhydrous DMSO (Life Technologies). The conjugate was prepared by mixing 1950 nmol of STh with 195 nmol of 8-arm PEG-SC. The final reaction volume was adjusted to 2 mL with PBS pH 7.2, and the reaction was carried out at room temperature with gentle stirring for 3 h. The reaction was stopped by adding excess 1 M Tris buffer (pH 8) and incubation for an additional 30 min to scavenge any unreacted NHS ester groups. The conjugate was dialyzed against PBS pH 7.2 overnight at 4°C using 10 kDa MWCO membrane (Spectrum Laboratories, Inc.). Furthermore, Vivaspin 500 columns (GE Healthcare) with MWCO 5 kDa were used to remove any unconjugated STh peptides, following the manufacturer's instructions.

2.4 | Amino acid analysis (AAA) of STh-8-arm PEG conjugate

Quantitative AAA was conducted at Pasteur Institute (France) as previously described[10] to estimate the concentration of STh in the STh-8-arm conjugate solution. Briefly, the conjugate (40 µL) underwent hydrolysis using 6 N HCl containing 1% phenol in glass tubes for 48 h at 110°C, along with a known quantity of the internal standard norleucine. Following the evaporation of HCl, amino acid composition analysis was conducted on the samples using an amino acid analyzer (Hitachi L-8800).

2.5 | Immunoblot analysis

To confirm the formation of the conjugate, an amount of conjugate containing 15 µg of STh (based on the AAA result, Figure S3) was resolved on a Mini-Protean TGX 4%–20% polyacrylamide gel (Bio-Rad Laboratories, Inc.) at 150 V for 45 min. The samples were then transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc.) for 20 min using the semidry method (Bio-Rad). The membrane was blocked with 3% milk in Tris-buffered saline-Tween 20 (TBST) for 1 h, washed 3 times and incubated overnight with a 1:3000 dilution of C30 anti-STp monoclonal antibody in 3% milk in TBST. Afterward, an anti-mouse IgG polyclonal antibody (HRP; VWR) at a 1:15000 dilution was incubated for 1 h. Finally, Clarity™ Western ECL substrate (Bio-Rad) was added, and the membrane was imaged using the ChemiDoc XRS + imaging system (Bio-Rad Laboratories).

2.6 | ELISA

To evaluate the impact of conjugation on the status of various epitopes on STh, an indirect ELISA was conducted using anti-STh polyclonal antibody and a panel of anti-ST monoclonal antibodies.[13] The ELISA protocol was similar to the competitive ELISA, with the exception that after blocking and washing with PBST, 120 µL of the indicated antibody (without competing peptide) was added. Goat anti-rabbit antibody (Sigma) (1:500) and rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) (1:4000) were used to detect the rabbit anti-STh polyclonal antibody and rabbit anti-mouse monoclonal antibodies, respectively.

2.7 | Toxicity analysis of STh-8-arm PEG conjugate

T84 cells (ATCC, Rockville, MD, USA) were seeded and grown to confluence on 48-well plates (Nunc, Roskilde, Denmark) in DMEM/F12 medium (Gibco Life Technologies), supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 0.2% gentamicin (Lonza). Cells were washed three times with 500 µL DMEM-F12 and preincubated with 80 µL DMEM-F12 containing 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 10 min at 37°C. Samples of the conjugate containing ~20 ng of STh or free STh was added to each well in duplicates and incubated at 37°C for 30 min. The reaction medium was then aspirated, and the cells were lysed with 0.1 M HCl at 20°C for 20 min. The lysates were centrifuged at 16,000 $\times g$ for 10 min, and the supernatants were collected for analysis. Levels of cGMP were determined using a cGMP ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) following the manufacturer's instructions.

2.8 | Mouse immunizations

Groups of five mice (Black 6) were subcutaneously immunized with STh-8-arm PEG conjugate containing an amount equivalent to 15 µg of STh or 15 µg free STh peptide (control). The ETEC double mutant heat-labile toxin (dmLT) adjuvant[36] (1 µg) was used in all immunization groups. Three booster doses were administered at 14-day intervals, and blood was collected on the 14th day after the final booster dose.

2.9 | Detection of serum anti-STh antibodies

Microtiter plates (Maxisorp NUNC-Immunoplate, Thermo Fisher Scientific) were coated with 50 µL/well of STh (1 µg/mL) in 10 mM PBS pH 7.2 and kept overnight at 4°C. The plates were then blocked with 1% ovalbumin in PBS and incubated for 2 h at 37°C. After blocking, the buffer was removed, and 50 µL/well of serum samples

(1:100 dilution in PBS) were added in duplicates and incubated for 2 h at 37°C. Following incubation, the plates were washed with PBST first and then with PBS three times. A secondary antibody (anti-mouse IgG antibody HRP conjugate, Sigma-Aldrich) diluted 1:10000 in PBST was added to each well (50 µL/well) and incubated for 2 h at 37°C. After incubation, the plates were washed again with PBST and PBS. Then, 50 µL/well of TMB substrate (3,3′,5,5′-tetramethylbenzidine, G-Biosciences) was added, and the plates were incubated at RT until the color developed. The reaction was stopped by adding 25 µL/well of 3 N H_2SO_4. Spectrophotometric readings were taken at 450 nm using a plate reader (Tecan infinite M200 Pro, Tecan Switzerland).

2.10 | Toxin neutralization assay

The neutralizing activity of mouse sera was determined by mixing individual mouse serum with native STh and adding the mixture to HEK293E cell line stably expressing human GC-C, as described previously.[37] Briefly, 20 µL mice serum was incubated with 10 nM STh overnight at 4°C in duplicate. The next day, the cells were treated with IBMX for 30 min, followed by a 30-min incubation with a mixture of the mouse sera and 10 nM STh. Cells were subsequently lysed in 100 µL of 0.1 N HCl, and cGMP was measured using radioimmunoassay (RIA) as described previously.[37]

3 | RESULT AND DISCUSSION

3.1 | Peptide expression and characterization

To prepare STh peptide for conjugation to the 8-arm PEG cross-linker, we expressed STh recombinantly. Following C18 reversed-phase chromatography, four distinct peaks were eluted at 44%, 48%, 51%, and 56% buffer B (Figure S1). The yields obtained (from 11 L medium) were 627, 748, 2838, and 671 µg for the 44%, 48%, 51%, and 56%, respectively. To identify the correctly folded STh isomer, competitive ELISA using C30 anti-STp mAb was conducted. The results indicated that the 51% peak peptide had an antigenicity very similar to synthetic STh,[11] suggesting correctly folded STh, unlike peptide from the other peaks, which all had drastically reduced antigenicity (Figure 1). MALDI-MS analyses of the peptides from the individual peaks revealed that all the four peaks contained masses corresponding to the theoretical masses of STh + H^+, STh + Na^+, and/or STh + K^+ (Table 1 and Figure S2). Because STh contains six cysteines that can potentially form multiple intramolecular disulfide bridges,[38] it is not unusual that multiple isomers form during recombinant production. This is consistent with previous results using the DsbC purification system.[11] A previous study also suggests that STh has the property of binding to metals such as zinc and iron,[39] corroborating our observation of STh binding to sodium and potassium adducts (Table 1, Figure S2). Notably, the correctly folded isomer, which has the highest yield, is the one used in conjugation experiment described below.

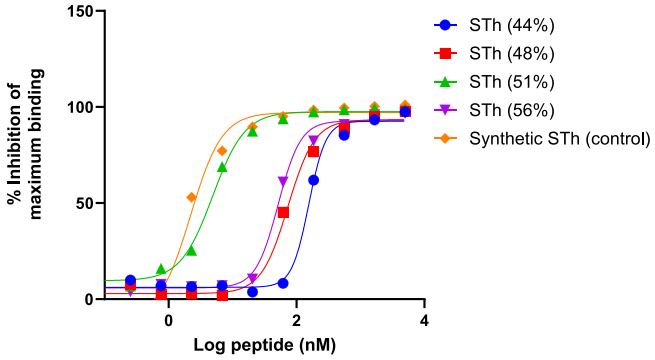

FIGURE 1 **Competitive ELISA.** Competitive ELISA using C30 anti-STp mAb was conducted to identify the correctly folded isomer following reversed-phase chromatography. The peptides were tested for competition at concentrations ranging from 5 to 0.085 µM.

TABLE 1 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) analysis of peaks detected following reversed-phase chromatography.

Peak	Expected mass (Da) $(M+H)^+$; 3 Cys-bridges	Observed masses (Da)
44%	2041.63	2041.574[a] 2063.564[b]
48%	2041.63	2041.584[a] 2063.564[b] 2079.562[c]
51%	2041.63	2041.608[a] 2063.586[b]
56%	2041.63	2041.599[a] 2063.588[b]

[a]STh + H^+.
[b]STh + Na^+.
[c]STh + K^+.

3.2 | STh readily reacts with NHS ester-activated multi-arm cross-linker

NHS esters find widespread application in conjugating proteins and peptides. They exhibit the capability to react with amines under mild alkaline pH conditions.[40] Within protein molecules, NHS ester cross-linking reagents primarily couple with the α-amines at the N-terminus and the ε-amines of lysine side chains.[34] Reaction with primary amines results in the formation of stable amide linkages.[34] In the present study, we used an NHS ester-activated 8-arm cross-linker, owing to the simple one-step conjugation process, combined with its ability to couple multiple STh molecules per cross-linker, as illustrated in Figure 2. Each arm reacts with the α-amines present on the N-terminus of STh (Figure 2A,B), ensuring it is defined in terms of its orientation, and the number of STh peptides per arm and per cross-linker. Notably, there were no signs of cloudiness or precipitation observed during the conjugation reaction, dialysis, or multiple freeze–thaw cycles following storage at −20°C. Previous research indicated

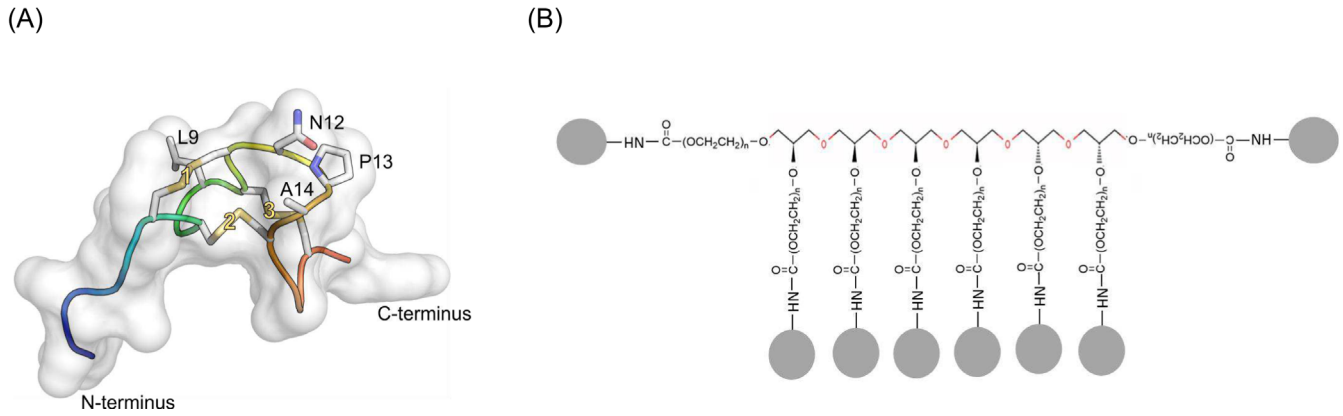

FIGURE 2 **Structural model of STh and chemical structure of the STh-8-arm PEG conjugate.** (A) The structural model of STh[12] is shown as a rainbow-colored cartoon within a transparent white surface representation. The amino group of the N-terminus is the site for chemical conjugation. The side chains of the three cysteine pairs that form the structurally defining disulfide bridges are shown as CPK-colored sticks (yellow numbers). Bridges 2 and 3 are shared with the uroguanylin and guanylin peptides, while bridge 1 is specific to the ST peptides. The side chains of residues important for toxicity are also shown as CPK-colored sticks. (B) Illustration of the chemical structure of STh-8-arm PEG. The gray circle depicts the STh peptide (not to scale).

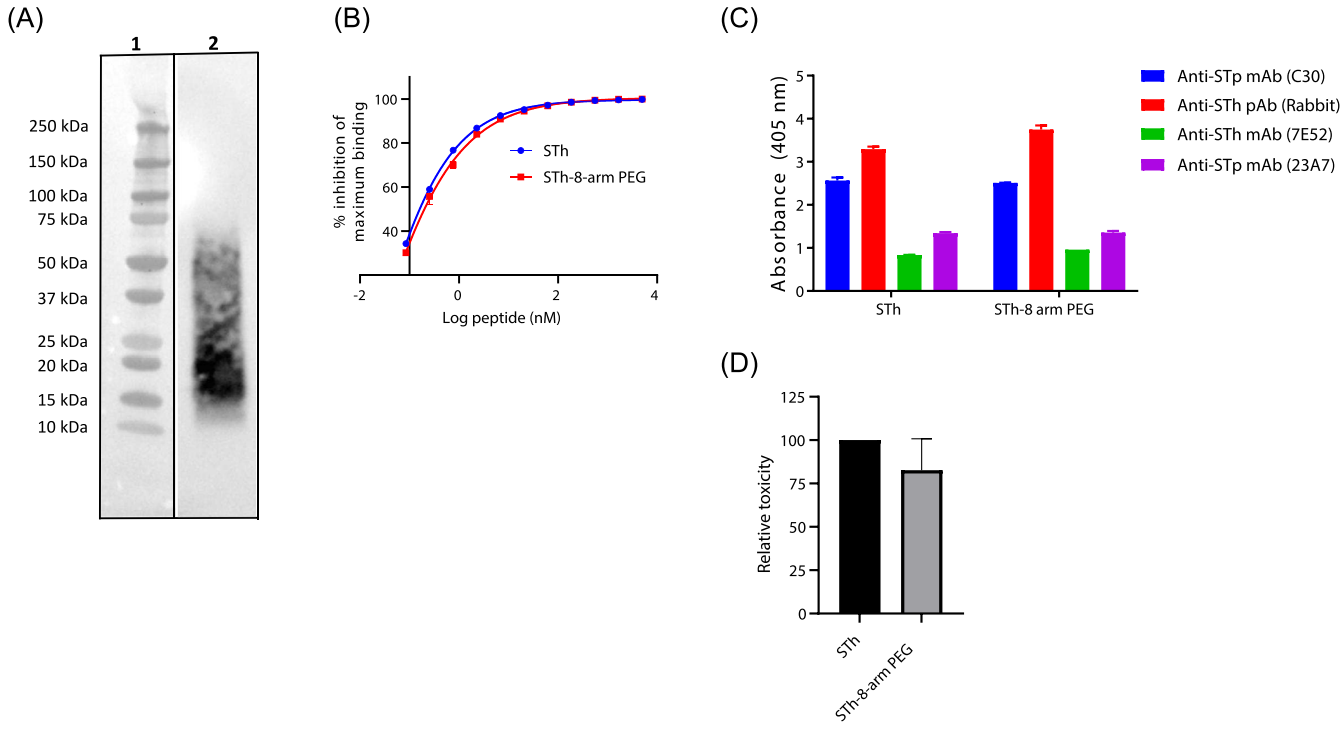

FIGURE 3 **Immunoblot, antigenicity and toxicity analyses of STh-8-arm PEG conjugate.** (A) Immunoblot analysis of STh-8-arm PEG. A sample (15 μg) of the conjugate was resolved on 4%–20% SDS-PAGE gel, transferred to a 0.45 μm nitrocellulose membrane, blocked, and detected with the C30 anti-STp mAb. Lane 1, molecular masses (kDa) of the protein standard; Lane 2, STh-8-arm PEG conjugate. (B) Competitive ELISA of STh peptide and STh-8-arm PEG conjugate. Nunc microtiter plates were coated with 100 μL of the STh-8-arm PEG conjugate amounting to ~4 ng of STh per well or free STh (control) for 40 min at RT, and competitive ELISA was conducted as detailed in Section 2.2. The IC_{50} values were calculated from the percentage inhibition of the maximum binding curves using four-parameter logistic regression analysis (Prism 9, GraphPad Software, La Jolla, CA) as done previously.[10] (C) Indirect ELISA. A panel of anti-ST monoclonal and a polyclonal anti-STh antibody was used to determine the overall status of the epitopes in the conjugate. The primary antibody concentrations used were as follows: C30 anti-STp mAb (1:16,000); 7E52 (1:100); 23A7 (1:100) and rabbit polyclonal antibody (1:2000).[13,15] (D) Toxicity analysis. The toxicity of STh-8-arm PEG (equivalent to 20 ng STh peptide) or free STh (20 ng) was analyzed in duplicate wells in a T84 cell assay, and the cGMP levels from each well were estimated using a cGMP ELISA kit. Relative toxicity values compared to the STh peptide are presented. Error bars in B, C and D indicate ±SD.

that conjugates formed by coupling STh through the N-terminus elicited antibodies with better neutralization properties compared to the one coupled through the C-terminus.[41] This is consistent with the fact that the C-terminus of STh contains the Tyr19 epitope that frequently elicits neutralizing antibodies, which do not cross-react with the endogenous ligands.[13] Therefore, it is suggested that the C-terminus of STh is left intact and exposed.[14,41]

The concentration of STh in the conjugate was determined to be 1.1 mg/mL, following AAA (Figure S3). To confirm the formation of the conjugate, an immunoblot was conducted using the C30 anti-STp monoclonal antibody. As shown in Figure 3A, STh-8-arm conjugate with a molecular weight of ~15–60 kDa was detected. Using a competitive C30 anti-STp monoclonal ELISA, we were able to further determine that the 50% inhibitory concentrations (IC_{50}) of the conjugate and STh were nearly equal, that is, ~15 nM (Figure 3B), suggesting correctly folded STh present in the conjugate. Furthermore, an indirect ELISA using anti-STh polyclonal antibody and three monoclonal antibodies indicates that the antigenicity of both coatings (free STh vs. conjugated STh) is near identical (Figure 3C). The antibodies we used, namely, clone 30, 7E52 and 23A7, predominantly recognize Tyr19, Glu 8, and Leu 9 residues in STh,[13,15] respectively. Collectively, the overall 3D structure of STh did not seem to be affected following the chemical conjugation (Figure 3C).

3.3 | STh-8-arm PEG conjugate retains toxicity

The toxicity of the conjugate was assessed using T84-cell assays. The T84-cell assay results showed that the toxicity of STh was retained after coupling (Figure 3D). This could be attributed to the fact that the individual STh peptides are linked to longer chains of PEG units via the N-terminus, which appears to provide flexibility and steric freedom to interact freely (and activate) with the GC-C receptors in the T84 cells, compared to conventional protein carrier-peptide conjugates. STh residues L9, N12, P13, and A14 are receptor-interacting[14,15] (Figure 2A) and do not seem to be affected by the N-terminal conjugation. Significant reduction in toxicity from conjugation observed in some conjugates[18,23] may not ensure vaccine safety, highlighting the need for detoxifying mutations.[14] In this regard, promising single mutants like STh-A14T, and STh-N12S,[16,21] show highly reduced toxicity[11] and elicit neutralizing antibodies.[10,16,19,21] Double mutants such as L9A/A14T and L9S/A14T also show no detectable toxicity and reduced cross-reaction.[10,11]

3.4 | STh-8-arm PEG conjugate is immunogenic

To evaluate the immunogenicity of the STh-8-arm PEG conjugate, subcutaneous immunization of mice with the conjugate equivalent to 15 μg of pure STh (based on AAA), premixed with 1 μg of dmLT adjuvant was conducted. The result indicated that anti-STh (IgG) antibodies were induced in those mice immunized with the conjugate but not in those mice immunized with dmLT-adjuvanted free STh (Figure 4A). Consistent with the level of anti-STh antibodies (Figure 4A), three of the five sera from STh-8-arm PEG immunized mice also exhibited a moderate level of neutralization activity against native STh toxin (Figure 4B). It is worth noting that STh is non-immunogenic per se, as evidenced by the absence of anti-STh antibodies in mice immunized with free STh peptide (plus dmLT adjuvant) (Figure 4), as well as from natural ETEC infections. Previous studies have indicated that despite high anti-STh titers achieved with peptide-protein carrier conjugates, some mice sera lack neutralizing

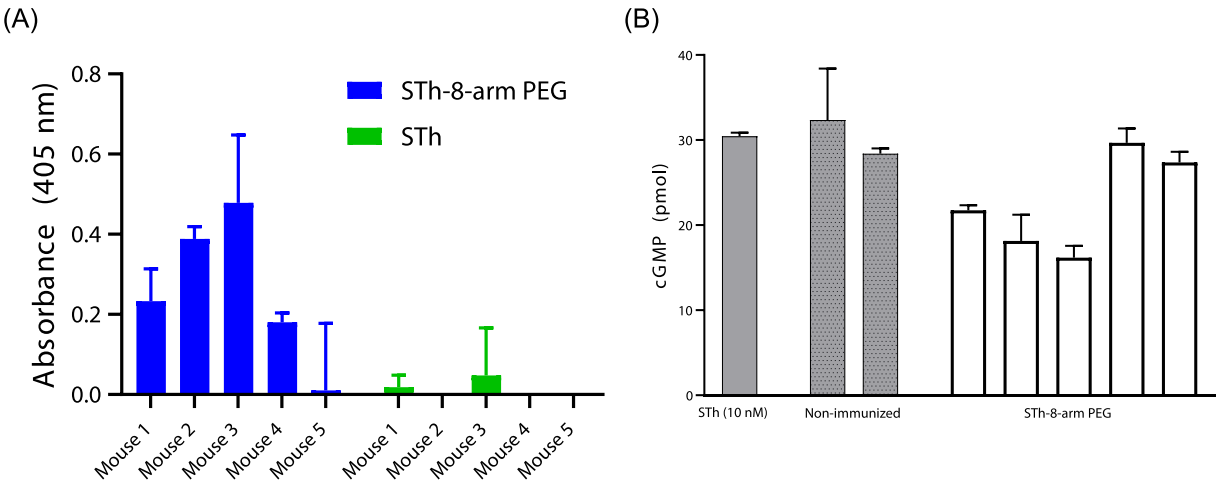

FIGURE 4 **Detection of serum anti-STh antibodies and toxin-neutralizing activity.** (A) Detection of serum anti-STh antibodies. Microtiter plates were coated with STh or without STh (PBS; blank wells), and 50 μL/well of serum samples (1:100 dilution in PBS) were added. The experiments were conducted in duplicates, and the absorbance from the blank wells was subtracted from wells coated with STh. Error bars indicate ±SD. (B) Neutralization of STh toxin by sera from immunized mice. The neutralizing activity of the mouse sera was assessed using HEK293E cell lines expressing the human GC-C receptor. Cells were treated with a mixture of mouse sera and 10 nM STh, and the released cGMP was quantified by RIA. Sera from nonimmunized mice were used as negative controls.

activity.[10] To the best of our knowledge, this is the first time that STh has been shown to elicit antibodies when coupled to a synthetic non-nanoparticle carrier. Previously, the only synthetic carrier, which led to neutralizing antibodies, was nanoparticulated ST-PLGA conjugate.[42] In that study, they immunized with an STh dose ~2.3 times higher than that used in the present study and employed a different immunization route, adjuvant, and regimen.[42]

PEGs have been commonly used in the pharmaceutical industry to modulate the pharmacokinetic and physicochemical properties of drug.[43] PEGylated substances exhibit reduced antigenicity, immunogenicity and toxicity, and enhanced circulation half-lives in vivo.[43,44] Multi-arm PEGs, in particular, have been widely utilized in hydrogel formation, as well as PEGylation of proteins and nanoparticles (reviewed in literature[45]). Synthetic platforms such as poly(amidoamine) (PAMAM) dendrimers also appear to be attractive alternatives for delivering peptide vaccine antigens, as they allow the conjugation of a high density of antigens and functionalization with desired reactive groups. However, the toxicity of the amine groups at physiological conditions limits their application.[46] In this regard, PEGylation of PAMAM has been shown to reduce toxicity.[46] Indeed, a dendrimer-conjugated peptide vaccine was found to elicit *Chlamydia*-specific serum antibodies in mice, which further provided protection of immunized mice against a challenge with *Chlamydia trachomatis*.[47]

4 | CONCLUSIONS

In the field of vaccine development, it is highly advantageous to utilize a simple hapten-carrier coupling method that yields a well-defined immunogen, considering both production cost and product consistency. This study demonstrates the potential of coupling the non-immunogenic STh with a multi-arm PEG cross-linker, which induced anti-STh antibodies in mice. While this discovery is promising as it introduces a new method to enhance peptide immunogenicity, further optimization is crucial to achieve robust neutralization activity. In this regard, synthetic multi-arm platforms are available in diverse molecular weights, arm lengths, and arm numbers. Moreover, there is a plethora of functional groups to select from, facilitating precise customization of peptide orientations on the carrier. Furthermore, these platforms provide the advantage of accommodating multiple peptide antigens, thereby enabling the development of multivalent vaccines targeting ETEC and other pathogens. The current research illustrated that haptens, like STh, when covalently linked to multi-arm PEGs, become immunogenic. On the other hand, our study also suggests that PEGylation of other substances such as drugs may also trigger the formation of antidrug antibodies, which underscores the need for careful consideration. Overall, our study contributes to the ongoing efforts in vaccine development by presenting a potential alternative approach for creating molecularly defined conjugates for vaccine and other applications, such as ELISA coating antigens.

AUTHOR CONTRIBUTIONS

Conceptualization: Ephrem Debebe Zegeye. *Methodology*: Ephrem Debebe Zegeye and Pål Puntervoll. *Formal analysis*: Ephrem Debebe Zegeye, Pooja Chaukimath, Yuleima Diaz, Sandhya S. Visweswariah, and Pål Puntervoll. *Investigation*: Ephrem Debebe Zegeye, Pooja Chaukimath, Yuleima Diaz, Sandhya S. Visweswariah, and Pål Puntervoll. *Supervision*: Ephrem Debebe Zegeye, Sandhya S. Visweswariah, and Pål Puntervoll. *Visualization*: Ephrem Debebe Zegeye, Pål Puntervoll, and Sandhya S. Visweswariah. *Writing—original draft preparation*: Ephrem Debebe Zegeye. *Project administration*: Pål Puntervoll and Sandhya S. Visweswariah. *Funding acquisition*: Pål Puntervoll and Sandhya S. Visweswariah. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

INSTITUTIONAL REVIEW BOARD STATEMENT

Mouse immunization procedures were carried out in agreement with the Control and Supervision Rules, 1998 of Ministry of Environment and Forest Act (Government of India), and the Institutional Animal Ethics Committee of the Indian Institute of Science (Approval CAF/Ethics/547/2017).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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