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(54) **Title:** MENINGOCOCCAL PROTEIN BASED VACCINE FORMULATIONS AND METHODS FOR MANUFACTURING THEREOF

(57) **Abstract:** Present invention provides fusion proteins with desired reduction in factor H binding, particularly the present invention provides optimized manufacturing process for fusion proteins and formulations comprising the fusion proteins. Present invention provides an efficient platform process for manufacturing an effective vaccine formulation against Neisseria meningitidis that meets multiple criteria including improved immunogenicity, safety, stability, and affordability.

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MENINGOCOCCAL PROTEIN BASED VACCINE FORMULATIONS AND METHODS FOR MANUFACTURING THEREOF

FIELD

The present invention relates to field of vaccine formulations and methods for manufacturing thereof. Particularly, the present invention relates to Upstream, Downstream and formulation development of *Neisseria meningitidis* (meningococcal) serogroup B based recombinant/ chimeric protein antigens, methods of preparing such chimeric protein-based formulations and use of these formulations for prevention and/ or treatment of subjects with *Neisseria meningitidis* (meningococcal) serogroup B infections.

BACKGROUND

All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

Neisseria meningitidis is an important pathogen, particularly in children and young adults. Septicemia and meningitis are the most life-threatening forms of invasive meningococcal disease (IMD). Case fatality rates remain around 10% for disseminated disease, while a third of survivors of meningococcal disease suffer from significant debilitating, and long-term sequelae. This disease has become a worldwide health problem because of its high morbidity and mortality.

Toxoid-based vaccines have almost eliminated diphtheria and tetanus in wealthy countries, while capsule-based vaccines have substantially reduced disease caused by Haemophilus influenzae, Streptococcus pneumoniae, and some strains of Neisseria meningitidis. However, challenges remain in developing vaccines against pathogens for which toxoid and capsule-based vaccines are not feasible. These pathogens include non-typeable strains of H. influenzae and S. pneumoniae, un-encapsulated pathogens such as Neisseria gonorrhoeae and Moraxella catarrhalis and encapsulated serogroup B N. meningitidis, for which a capsule-based vaccine is not feasible. Given the rise in the emergence of multi-drug resistant bacteria, new approaches for vaccine development are required. The development of a vaccine against

serogroup B meningococcus presents particular difficulties because the polysaccharide capsule is poorly immunogenic owing to its immunologic similarity to human neural cell adhesion molecule. Also, strategies for generating successful vaccines are hampered by pathogen diversity and the difficulties associated with presenting epitopes from membrane-embedded surface proteins to the immune system.

Neisseria meningitidis (N. meningitidis) is a Gram-negative bacterium which colonizes the human upper respiratory tract and is responsible for worldwide sporadic and cyclical epidemic outbreaks of, most notably, meningitis and sepsis. N. meningitidis typically possess a cytoplasmic membrane, a peptidoglycan layer, an outer membrane which together with the capsular polysaccharide constitute the bacterial wall, and pili, which project into the outside environment. Neisseria meningitidis (Nm) remains a leading cause of sepsis and bacterial meningitis in children and young adults. There are approximately 500,000 cases of meningococcal disease each year with around 50,000 deaths. In developed countries, the bacterium is a leading cause of mortality among children, has important public health impacts during outbreaks in schools and universities, and can cause profound disability in survivors. Encapsulated strains of N. meningitidis are a major cause of bacterial meningitis and septicemia in children and young adults. N meningitidis can be classified into at least 12 serogroups (including serogroups A, B, C, H, I, K, L, 29E, W135, X, Y and Z) based on chemically and antigenically distinctive polysaccharide capsules. The most common serogroups being A, B and C which are responsible for 90% of disease worldwide. Serogroup B is the most common cause of meningococcal disease in Europe, USA and several countries in Latin America and causes epidemics in sub-Saharan Africa every 5-10 years. Meningococcal is a devastating disease that can kill children and young adults within hours despite the availability of antibiotics. Therefore, prophylactic immunisation is the best way to protect individuals from meningococcal infection.

Vaccines are available based on the bacterial polysaccharide capsule, but the polysaccharide capsule of N. meningitidis serogroup B is poorly immunogenic as it has structural identity with a human glycoprotein in neural tissue and could induce autoimmunity if used as a vaccine. Therefore, serogroup B 'humanization' put on-hold early efforts towards the development of a safe and immunogenic conjugate polysaccharide vaccine against B group since the polysaccharide does not elicit serum bactericidal antibodies and in vitro anti-capsule B antibodies recognize neural cell adhesion molecules in fetal brain tissue.

Two main approaches have been used to develop vaccines against serogroup B N. meningitidis; outer membrane vesicle vaccines (OMVV) and recombinant protein subunit vaccines. OMVVs were first developed in the 1980s. The immunodominant antigen in meningococcal OMVVs is PorA, an abundant outer-membrane porin with eight surface-exposed loops. Loops one and four are termed variable region 1 and 2 (VR1 and VR2), respectively, as they generate immune responses and are subject to antigenic variation. The VR2 loop dominates PorA-specific immunity elicited by OMVVs, which offer limited or no cross-protection against strains expressing PorA with a different VR2. When longer follow up periods were assessed including an OMV vaccine studied over 20 months in Chile, the efficacy decreased to 50%, indicating poor longevity of response (A.L. Wilkins, M.D. Snape et al 2017).

To broaden coverage, OMVVs containing multiple PorAs have been developed and selected for the prevalence of PorA sequences in circulating strains. However, OMVVs present complex manufacturing and regulatory issues. Prior art vaccines have often made use of purification of so called "blebs" which represent vesicles shed from the cell surface of the particular organism of interest. However, such a crude product carries many problems. For example, there is wide variation in the composition of these blebs. There is no reliable way of controlling which proteins are included or excluded from these blebs. These blebs may or may not include polysaccharide- coating elements of the organism of interest. The proportions of the various components of the blebs in relation to one another cannot be reliably determined. The composition of these blebs cannot be easily determined or controlled.

The OMV in Bexsero® (GSK Vaccines) is highly reactogenic, so the vaccine is routinely given with paracetamol due to which parents become concerned about adverse effects (Prymula, R. 2014). Bexsero® provides uncertain coverage as 1) antigens are derived from a single meningococcal strain (Tan, L., et al. 2010) 2) most studies of immunogenicity were done with a 3+1 schedule and not the 2+1 schedule planned for the UK (doses at 2, 4 and 12 months) 3) indirect not direct correlates of protection have been mostly measured in 13 month olds, and not in infants who are at the greatest risk.

N. meningitidis serogroup B vaccines based on Outer membrane vesicle (OMVs) have been used successfully to prevent epidemics (most recently in New Zealand) but only confer protection against strains expressing the same variant of PorA (an outer membrane porin) as

in the OMV. OMVs as immunogens are not favoured because consistency and toxicity can be problematic during manufacture. For example, OMVs may contain toxic lipopolysaccharide (LPS).

PorA is the most abundant meningococcal outer membrane protein (OMP), which also elicits SBA, and is the main target of immune responses elicited by OMV vaccines which have been used successfully in outbreaks. PorA variants differ in their variable regions (VRs), surface exposed loops which are the target of immune responses. Of note, VR2 is responsible for most SBA elicited by OMV vaccines. However, PorA is an integral OMP with multiple hydrophobic domains. This makes PorA difficult to produce as a recombinant protein in its native conformation, limiting its use as an antigen in subunit vaccines.

Factor H binding protein (fHbp) is also an antigen that elicits serum bactericidal antibody responses in immunised individuals and is a key component of investigational vaccines for the prevention of meningococcal, in particular serogroup B, disease that are currently being evaluated in clinical trials. Factor H Binding Protein (fHbp, also referred to in the art as lipoprotein 2086 (Fletcher et al (2004) Infect Immun 72:2088-2100), Genome-derived Neisserial antigen (GNA) 1870 (Masignani et al. (2003) J Exp Med 197:789-99) or "741") is a surface-exposed lipoprotein expressed in the N. meningitidis bacterium. Based on differences in the nucleotide and predicted amino acid sequences, fHbps from different Neisseria meningitidis strains have been categorised using several schemes. These include two subfamilies (A and B) (Murphy E, et al. (2009) The Journal of Infectious Diseases 200: 379- 389) or three variant groups (V1, V2, and V3) (Masignani V, et al. (2003) The Journal of Experimental Medicine 197: 789-799), with subfamily A corresponding to V2 and V3, and subfamily B corresponding to V1 (which is the most abundant).

The recombinant subunit vaccines Bexsero® (GSK vaccines) and Trumenba® (Wyeth Pharmaceuticals) contain an important meningococcal antigen, factor H binding protein (fHbp), which is a lipoprotein composed of two β-barrels that tightly bind domains 6 and 7 of human complement factor H (CFH). fHbp is antigenically variable; databases of genome sequences contain more than 900 different fHbp peptides, which fall into three variant groups or two subfamilies: V1 (subfamily B), V2 and V3 (both subfamily A). In general, immunisation with a particular fHbp induces cross-protection against strains that express fHbp belonging to the same, but not a different, variant group, although there can be cross-protection between fHbp variant groups 2 and 3 (subfamily A). Bexsero® contains a single

fHbp peptide (V1.1), with two other recombinant antigens as well as an OMV, while Trumenba®is composed solely of two fHbp peptides (V1.55 and V3.45). To date, no vaccine studies have included V2 fHbp even though strains expressing this variant account for around 20-30% of all isolates (38% of UK cases). There is no vaccine with a v2 fHbp as this is an inherently unstable antigen. fHbp on Neisseria meningitidis is a 27 KDa lipoprotein that consists of two beta barrels (an N terminal barrel and a C terminal barrel) joined by a short amino acid linker. The reason for the lack of V2 fHbp in current licensed vaccines is the instability of its N terminal β-barrel (Prymula, R. 2014; Johnson, S 2012). Further, protein stability is important during vaccine manufacturing as it affects yield and is a significant issue for quality control. Also, antigens in Bexsero® and Trumenba® have exact sequence matches to 36 and 4.8%, respectively of serogroup B N. meningitidis disease isolates currently circulating in the UK, leading to concerns about their ability to provide broad coverage against an antigenically diverse pathogen. Further, modified V2 fHbp having increased stability over wild type V2 fHbp has been disclosed wherein modified V2 fHbp comprising atleast six mutations at Ser35, Aspl07, Vall l2, Leul l4, Serl37 and Glyl38. Since, a single fHbp does not provide universal protection against meningococcal disease, therefore, immunisation with a vaccine comprising representatives from each of the three variants, VI, V2 and V3, is necessary for a broad-N meningitidis serogroup B protection.

Both Bexsero® and the Trumenba® vaccine(s) were developed before it was appreciated that Complement factor H (CFH) binds the meningococcus via fHbp at high affinity, and that this can impair immune responses (Schneider, M 2009). Although fHbp has been demonstrated to be an important protective antigen, the extent to which fHbp interacts with fH upon immunization, and whether any fHbp-fH interaction affects the overall immunogenicity of fHbp in humans is currently unknown. Studies in hfH transgenic mice and infant rhesus macaques, the latter having a polymorphism in the fH gene that allows for either high or low binding to fHbp (Konar M 2015), have demonstrated that binding of fH to fHbp lowers the immunogenicity of fHbp (Beemink PT 2011) (Costa 2014) (Giuntini S 2015) (Granoff DM 2015) (Rossi R 2013).

Further, Upstream, Downstream and formulation development can often be the rate-limiting step in early introduction of biopharmaceuticals into the market and in meeting the demands of the population.

Upstream process development includes scale-up of a fermentation process to ensure a similar product yield with quality at large scale as is produced at small scale. Various cultivation parameters, such as media composition, pH, agitation, aeration, temperature, cell density, the concentration of inducers, induction time, and feeding strategies affect the protein expression level depending upon expression systems. Thus, it is essential to evaluate each of the cultivation conditions for the expression of every recombinant protein and the development of effective bioprocesses.

Escherichia coli is the most widely used bacterial host for the production of recombinant proteins due to: (1) its fast growth rate with a generation time spanning 20 min under optimized conditions (Clark and Maaløe, 1967), (2) well-developed tools of molecular manipulations along with in-depth knowledge of its biology, and (3) the ability to achieve high cell density using inexpensive culture reagents. But in practice, a number of obstacles encountered along the pipeline must be overcome. These include poor growth of the host strain, protein instability or toxicity, aggregation and inclusion body formation, unsuitability of environmental conditions (temperature, pH, salt concentration, etc) and even no amplified expression at all. When the protein of interest cannot be detected or it is detected but at very low levels (less than micrograms per liter of culture), this could be due to slower growth rate, low final cell density, and death. E. coli cannot perform post-translational modifications, limiting the product range that can be produced in a soluble and active form in this host organism. Furthermore, E. coli cannot secrete recombinant proteins. Consequently, recombinant E. coli cells need to be disrupted to access the intracellular product, which is then usually purified by several steps of filtration.

In addition, high level expression of recombinant proteins in E. coli results in aggregation of expressed proteins into inclusion bodies (IB's). This poses a serious challenge for producing soluble recombinant proteins with proper biological function at the industrial scales, as it requires extensive processing involving isolation from cell, solubilization, refolding and purification to produce the bioactive proteins. Altering culture conditions usually present the simplest solution to reduce IBs formation in E. coli. However, culture conditions favourable for soluble protein production may vary depending on the involved proteins of interest and the used host strains of E. coli, and thus require experimental optimization. Factors such as expression strain, fermentation medium, and operating conditions, all play important roles in the process upscaling to maintain or improve yield on a larger scale and eventually at an industrial scale to provide large quantities of the protein through a cost-effective,

commercially viable manufacturing process. It is important, therefore, to identify an appropriate parameter or set of parameters that will be critical to the specific process.

High-level production of recombinant proteins will subsequently require an efficient purification process at the industrial scales since it contributes to the approval of therapeutic products for human use. Cell disruption is required for recovery of the desired proteins, expressed as intracellular IBs. Cell disruption can be very effective in small scale work; however, upscaling is very poor. Sonication has high energy requirements, as well as high health and safety issues, due to noise. It is not continuous. Chemical Cell Lysis pose significant health and safety risks to the user and cost of using large volumes of reagents needed for large-scale production can be prohibitive. Further, the presence of salts and detergents may not be compatible with protein assays. It may also affect the results of downstream applications (e.g., mass spectrometry). Hence, appropriate cell lysis method needs to be considered for optimum results during upscaling.

It is well-established that an increased product concentration in the upstream process leads to a higher volume of chromatography resin and a higher buffer requirement. Host cell proteins (HCPs) and DNA are the main source of impurities, and the HCPs of each process vary significantly from each other in their molecular mass, charge, hydrophobicity, and structure. Therefore, they present a challenge for chromatographic purification.

Where a purified soluble active recombinant protein is needed, it is invaluable to have means to (i) detect it along the expression and purification scheme, (ii) attain maximal solubility, and (iii) easily purify it from the E. coli cellular milieu. The expression of a stretch of amino acids (peptide tag) or a large polypeptide (fusion partner) in tandem with the desired protein to form a chimeric protein may allow these three goals to be straightforwardly reached. Being small, peptide tags are less likely to interfere when fused to the protein. However, in some cases they may provoke negative effects on the tertiary structure or biological activity of the fused chimeric protein. Hence, peptide tags should be removed too because it can affect protein conformation, hamper the interaction with a partner molecule or decrease the biological activity. Indeed, when these tags are removed, the final solubility of the desired product is unpredictable. In the case of tag removal by enzyme digestion, expression vectors possess sequences that encode for protease cleavage sites downstream of the gene coding for the tag. Choosing among the different proteases is based on specificity, cost, number of amino acids left in the protein after cleavage and ease of removal after digestion C-type

cysteine protease from Tobacco Etch Virus (TEV) is among the most widely used. However, expression of TEV protease in Escherichia coli faced difficulties regarding protein yield (product yield is reduced) or low solubility of the protein at the industrial scales which means that large volumes and often long incubation times are required for efficient cleavage.

Vaccines based on recombinant protein antigens generally require an adjuvant to achieve protection from the associated disease. Aluminum salts are the most prevalent adjuvants in vaccines approved for human use by the U.S. Food and Drug Administration. The point of zero charge (PZC) of the adjuvant is the point at which the net surface charge is zero; the PZC for aluminum oxyhydroxide is approximately 11, whereas the PZC for aluminum phosphate is approximately 4–5.5. Protein adsorption to the adjuvant surfaces is generally maximized when the sign of the net charge of the protein is opposite that of the adjuvant surface, allowing for an electrostatic attraction. Therefore, the protein vaccine formulation is formulated with adsorption buffers to improve adsorption to the surface. However, protein conformation can change when proteins bind to liquid-solid interfaces. Furthermore, conformational changes induced by binding to adjuvant could alter protein stability during long-term storage. For example, if adsorption is essentially complete, aggregation via pathways that occur in bulk solution are not likely to occur. Conversely, unfolding upon binding may expose normally buried residues to solvent, promoting degradation processes such as oxidation. (J Pharm Sci. 2009 September; 98(9): 2970–2993).

Physical and covalent stabilization upon long-term storage of recombinant protein antigens adsorbed to the adjuvant surfaces is of fundamental concern because of the potential impact of degradation on immunogenicity, toxicity, and efficacy of the recombinant protein antigens.

There are various factors affecting stability of recombinant protein antigen which include percentage of antigen adsorption on to the adjuvant surfaces, zeta potential, viscosity, solute concentration, pH changes, and temperature that contribute to storage stability.

Zeta potential is one such physical property which is essential to optimize the formulations of suspensions, emulsions and protein solutions, and predict interactions with surfaces. Zeta potential is exhibited by any particle in suspension, macromolecule or material surface. Knowledge of the zeta potential can reduce the time needed to produce trial formulations. It can also be used as an aid in predicting long-term stability. In certain circumstances, the particles in dispersion may adhere to one another and form aggregates of successively

increasing size, which may settle out under the influence of gravity. Further it can be seen that the zeta potential depends on the nature of the buffer used.

There are various factors affecting zeta potential which include excipient used in formulation, excipient compatibilities, concentration of a formulation components, pH and conductivity.

The variation in pH influences the electrostatic force, whereby the van der Waals forces remain constant for a given system. At a pH near the isoelectric point charge-charge repulsion is minimal between neutral molecules and attractive forces dominate resulting in a flocculated system, high viscosity and where the aggregation is most likely. This pH-effect was observed by Liu et al. and Chari et al. in antibody solutions.

The impact of pH and temperature on the stability and activity of recombinant protein antigens is factored by possible changes in the characteristics of recombinant protein antigens that include gradual aggregation, degradation processes such as oxidation affecting both physical and chemical stability.

Hence, formulation development becomes altogether more important for long-term storage of recombinant protein antigens. It is known that as aggregation increases, the effective concentration of available immunogen decreases. Therefore, a need exists for formulations and methods which overcome the problem of aggregation by stabilizing recombinant proteins against aggregation.

Accordingly, there is a need for vaccine against *Neisseria meningitidis* which has maximum adsorption of recombinant protein antigens to the adjuvant surfaces, shows low viscosity, is devoid of aggregation, has long-term stability across wide temperature ranges, has improved immunogenicity and at the same time is affordable and safe.

Also, there is a need for an efficient platform process for manufacturing an effective vaccine against *Neisseria meningitidis* that meets multiple criteria including improved immunogenicity, safety and affordability, in particular an improved formulation showing low viscosity, devoid of aggregation, and showing long-term stability across wide temperature ranges.

Also, there is a need to prepare chimeric proteins that are soluble, high yielding, and stable, wherein all requisite protein antigens are immunogenic.

To overcome the aforementioned limitations of prior art, applicant proposes improved Upstream, Downstream methods and formulation development of *Neisseria meningitidis* (meningococcal) serogroup B based chimeric protein antigens, methods of preparing such chimeric protein-based formulation.

SUMMARY

Applicant provides 1) Chimeric antigens (ChAs) against serogroup B N. meningitidis (MenB). ChAs exploit fHbp (non-lipidated) as a molecular scaffold to present the surface exposed PorA VR2 loop, which is achieved by inserting the VR2 loop ("10-20 amino acid" PorA VR2 loops instead of a "whole PorA protein) into a β-turn region in fHbp. ChAs retain epitopes from both fHbp and PorA and are found to elicit functional immune responses against both antigens. The integration of a VR2 loop does not alter the overall architecture of fHbp and that the VR2 loop folds into a conformation recognised by a bactericidal mAb. 2) Chimeric proteins that are soluble and high yielding, are stable, wherein fHbp and the PorA VR2 loop both are immunogenic. 3) Generate Chimeras composed of the most prevalent fHbp and PorA antigens to maximize vaccine coverage as Chimera composition mirrors the prevalent fHbp and PorA antigens circulating within a given geographical area 4) Insertion of PorA loop at a specific position in fHbp for obtaining desired reduction in Factor H binding (reduced by at least 10 %, at least 50%; preferably >70% as compared to wild type) & having Molecular weight in the range of 20 kDa to 40 kDa while preserving immunogenic epitopes of both fHbp & PorA.

Table-1: fHbp-PorA chimeras

Sr. No. of Chimera	Description of chimera	MW in kDa
Chimera 1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	20 - 40
Chimera 2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	20 - 40
Chimera 3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	20 - 40
Chimera 4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	20 - 40
Chimera 5	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	20 - 40

5) Inserting one PorA loop into one fHbp (instead of 2 PorA loops on one fHbp) & insertion of maltose-binding protein (MBP) tag along with His-tag to ensure optimum expression, solubility & stability. 6) Inserting TEV cleavage site in between His-MBP tags and chimera, to ease removal of tag during downstream processing & optimized Substrate (tagged recombinant protein/ modified fHbp fusion protein) to enzyme ratio (20:1 as compared to 5:1, 10:1), temperature/ incubation (30 °C, 15-18 hours). 7) Retention of plasmid for at least 50 generations that too without need for any antibiotic. 8) Using a combination of chemical lysis and homogeniser (instead of sonication or only chemical lysis) for large scale lysis of cells (Pressure-1000-1500 bar; 3-8 cycles) considering the advantages of using homogeniser include destruction of cell walls at 4-15 °C, effective for neutral lipid extraction, easy to use with smaller volumes, faster processing, reliable operation, constant shear rate, and fully scalable. 9) Yield of tagged fHbp protein could be at least 700mg of purified tagged protein from 100 grams of wet cell mass (Harvest) due to use of optimal concentration of inducer (IPTG (1 mM to 10 mM) or Lactose (1 g/L to 50 g/L)); use of Modified M9 salt media (Chemically defined media)/ Fed Batch Mode supplemented with L-Methionine (maintaining the L-Methionine concentration between 1-10 mM, preferably 2-5 mM, as compared to Luria Broth (LB) Media (Complex media)/ Batch Mode; stopping glucose feed and starting Glycerol feed when OD at 590/ 600 nm is ~ 20-100 and inducing the culture by adding and/or maintaining Lactose at 1-50 g/L in Fed batch mode. 10) Using two step chromatography (Ion Exchange followed by Affinity) instead of previously known 3 step chromatography (multistep Affinity chromatography, Ion Exchange chromatography followed by Size Exclusion). 11) uses stabilised V2 fHbp by substituting amino acids in its N-terminal β barrel (i.e. altering < 5% residues of this barrel). 12) Formulation comprising of Adjuvant (Alum hydroxide), Phosphate buffer, Mannitol & Polysorbate (Polysorbate 20) thereby imparting optimum pH (7-8), Osmolality (200-500, preferably 200-400 mOsmol/kg) and Zeta potential (-16 to -30 mV) resulting in maximum adsorption & preserving integrity/stability of chimeric proteins. 13) For TEV cleavage, Enzyme: Substrate ratio of 1:20 incubated for 18 hours are found to be optimum.

DETAILED DECEPTION OF THE DRAWING

The present invention will now be described with the help of the accompanying drawing, in which:

Figures 1-5 illustrate the vector map for the recombinant proteins/ modified fHbp fusion proteins in accordance with the embodiments of the present invention;

Figure-6 illustrates the vector map for TEV protease in accordance with an embodiment of the present invention;

Figure-7 illustrates the flow chart for seed development for 10 L scale fermentation batch for the production of tagged fHbp protein;

Figure-8 illustrates the flow chart for the production of tagged fHbp protein at 10 L scale fermenter;

Figures 9a-9h illustrate the growth profile for the tagged fHbp proteins at 10 L scale fermentation batch along with the SDS PAGE gel images;

Figure-10 illustrates the flow chart for seed development for 10 L scale fermentation batch for the production of TEV protease;

Figure-11 illustrates the production of TEV protease at 10 L scale fermenter;

Figures 12a-12b illustrate the growth profile for the TEV protease at 10 L scale fermentation batch along with the SDS PAGE gel image;

Figure-13 illustrates the percentage lysis obtained using homogenization;

Figures 14a-14b illustrate the SDS-PAGE image of TEV protease cleavage reactions using different substrate: enzyme ratios;

Figure-15 illustrates the Densitometric analysis of TEV protease cleavage reactions using different substrate: enzyme ratios;

Figure-16 illustrates the SDS-PAGE image of TEV protease cleavage reactions using different temperatures;

Figure-17 illustrates the Densitometric analysis of TEV protease cleavage reactions using different temperatures;

Figure-18 illustrates the flow chart for the purification of TEV protease by Ni-Sepharose Resin (pH 7.4);

Figure-19 illustrates the flow chart for the purification of TEV protease by Ni-Sepharose Resin (pH 8.5);

Figures 20a-20b illustrate the purification profiles of TEV protease at pH 8.5 and pH 7.4, respectively;

Figure-21 illustrates the flow chart for the purification of tagged proteins by Ni-Sepharose Resin;

Figure-22 illustrates the flow chart for the purification of tagged proteins expressed in inclusion bodies;

Figure-23 illustrates the flow chart for removal of tags from purified protein;

Figure-24 illustrates the flow chart for ion exchange chromatography step for purification of recombinant protein/ modified fHbp fusion protein;

Figure-25 illustrates the flow chart for affinity chromatography step for purification of recombinant protein/ modified fHbp fusion protein;

Figure-26 illustrates the graphical representation with individual data points and GMT highlighted for each day of bleeding;

Figure-27 illustrates the representative data for binding of human complement factor-H to wildtype fHbp as compared to Men B chimeric proteins;

Figure-28 illustrates the graphical representation with GMT at each time point (sera bleeding day) for MenFive serogroups;

Figure-29 illustrates the graphical representation with GMT at each time point (sera bleeding day) for MenB serogroups; and

Figure-30 illustrates the standard curve for protein estimation using Micro Bicinchoninic acid (BCA) method .

OBJECTS

Some of the objects of the present invention, which at least one embodiment herein satisfies, are as follows:

An object of the present disclosure is to provide an efficient platform process for manufacturing an effective vaccine formulation against *Neisseria meningitidis* that meets multiple criteria including improved immunogenicity, safety and affordability.

Another object of the present disclosure is to provide an efficient platform process for manufacturing an effective vaccine formulation comprising of one or more recombinant protein/ modified fHbp fusion protein derived from Neisseria meningitidis serogroup B and pharmaceutically acceptable carrier or excipient.

Another object of the present disclosure is to develop and optimize upstream bioprocess to increase cell density and recombinant protein/ modified fHbp fusion protein productivity for the lead cell lines.

Another object of the present disclosure is to develop and optimize downstream bioprocess for production of recombinant protein/ modified fHbp fusion protein with high yield and high purity.

Another object of the present disclosure is to develop and optimize formulation comprising recombinant protein/ modified fHbp fusion protein showing improved immunogenicity, low viscosity, devoid of aggregation; showing long-term stability across wide temperature ranges.

Another object of the present disclosure is to develop and optimize formulation comprising chimeric antigens (ChAs) against serogroup B N. meningitidis. ChAs exploit fHbp (non-lipidated) as a molecular scaffold to present the surface exposed PorA VR2 loop, which is achieved by inserting the VR2 loop (instead of a "whole PorA protein) into a β-turn region in fHbp. ChAs retain epitopes from both fHbp and PorA and are found to elicit functional immune responses against both antigens wherein the integration of a VR2 loop does not alter the overall architecture of fHbp and that the VR2 loop folds into a conformation recognised by a bactericidal mAb.

Another object of the present disclosure is to develop and optimize formulation comprising chimeric proteins that are soluble and high yielding, are stable, wherein fHbp and the PorA VR2 loop both are immunogenic.

Another object of the present disclosure is to develop and optimize formulation comprising Chimeras composed of the most prevalent fHbp and PorA antigens to maximize vaccine coverage.

Another object of the present disclosure is to develop and optimize formulation comprising chimeric proteins having Molecular weight in the range of 20 kDa to 40 kDa while preserving immunogenic epitopes of both fHbp and PorA:

Table-2: fHbp-PorA chimeras

Sr. No. of Chimera	Description of chimera	MW in kDa
Chimera 1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	20 - 40
Chimera 2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	20 - 40
Chimera 3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	20 - 40
Chimera 4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	20 - 40
Chimera 5	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	20 - 40

Another object of the present disclosure is to develop and optimize downstream comprising inserting TEV cleavage site in between His-MBP tags and chimera, to ease removal of tag during downstream processing and optimized Substrate to enzyme ratio/ temperature/ incubation, retention of plasmid for maximum generations that too without need for any antibiotic.

Another object of the present disclosure is using a combination of chemical lysis and homogeniser (with optimized conditions instead of sonication or only chemical method) for large scale lysis of cells.

Another object of the present disclosure is developing high yield of chimeric fHbp-PorA proteins due to use of optimal concentration of inducer; use of Modified M9 salt media (Chemically defined media)/ Fed Batch Mode supplemented with L-Methionine; stopping glucose feed and starting Glycerol feed when reaching a specific OD at 590/600 nm and inducing the culture by adding Lactose or IPTG.

Another object of the present disclosure is developing two step chromatography instead of previously known methods using at least 3 step chromatography.

Another object of the present disclosure is to develop and optimize formulation comprising Chimeric proteins, Adjuvant (Alum hydroxide), Phosphate buffer, Mannitol & Polysorbate (Polysorbate 20) thereby imparting optimum pH, osmolality and Zeta potential resulting in maximum adsorption & preserving integrity/ stability of chimeric proteins.

Still another object of the present disclosure is to provide a method of vaccinating a host.

Other objects and advantages of the present disclosure will be more apparent from the following description, which is not intended to limit the scope of the present disclosure.

DESCRIPTION

Before the present compositions and formulations of the invention are described, it is to be understood that this invention is not limited to particular compositions and formulations described, since such compositions and formulation may, of course, vary. It is also to be understood that the terminology used herein is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Although the present disclosure may be susceptible to different aspects and embodiments, certain embodiments are shown in the drawing and following detailed discussion, with the understanding that the present disclosure can be considered an exemplification of the principles of the disclosure and is not intended to limit the scope of disclosure to that which is illustrated and disclosed in this description.

Aspects and Embodiments are provided so as to thoroughly and fully convey the scope of the present disclosure to the person skilled in the art. Numerous details are set forth, relating to specific components, and processes, to provide a complete understanding of embodiments of the present disclosure. It will be apparent to the person skilled in the art that the details provided in the embodiments should not be construed to limit the scope of the present disclosure. In some embodiments, well-known composition, well-known processes, and well-known techniques are not described in detail.

The terminology used, in the present disclosure, is only for the purpose of explaining a particular embodiment and such terminology shall not be considered to limit the scope of the

present disclosure. As used in the present disclosure, the forms “a”, “an”, and “the” may be intended to include the plural forms as well, unless the context clearly suggests otherwise.

The terms "comprises”, “comprising”, “including” and “having” are open ended transitional phrases and therefore specify the presence of stated features, integers, steps, operations, elements, modules, units and/or components, but do not forbid the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. The particular order of steps disclosed in the process of the present disclosure is not to be construed as necessarily requiring their performance as described or illustrated. It is also to be understood that additional or alternative steps may be employed.

The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including", "includes" or "containing", "contains", and are inclusive or open -ended and do not exclude additional, non-recited members, elements or method steps. It will be appreciated that the terms "comprising", "comprises" and "comprised of" as used herein comprise the terms "consisting of", "consists" and "consists of". More specifically, the term “comprise” as used herein means that the claim encompasses all the listed elements or method steps, but may also include additional, unnamed elements or method steps. For example, a method comprising steps a), b) and c) encompasses, in its narrowest sense, a method which consists of steps a), b) and c). The phrase "consisting of" means that the composition (or device, or method) has the recited elements (or steps) and no more. In contrast, the term “comprises” can encompass also a method including further steps, e.g., steps d) and e), in addition to steps a), b) and c).

The terms first, second, third, etc., should not be construed to limit the scope of the present disclosure as the aforementioned terms may be only used to distinguish one element, component, region, layer or section from another component, region, layer or section. Terms such as first, second, third etc., when used herein do not imply a specific sequence or order unless clearly suggested by the present disclosure.

Furthermore, the terms "first", "second", "third" or "(a)", "(b)", "(c)", "(d)" etc. and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein. In case the terms "first", "second", "third" or “(A)”, “(B)”

and “(C)” or "(a)", "(b)", "(c)", "(d)", "i", "ii" etc. relate to steps of a method or use or assay there is no time or time interval coherence between the steps, that is, the steps may be carried out simultaneously or there may be time intervals of seconds, minutes, hours, days, weeks, months or even years between such steps, unless otherwise indicated in the application as set forth herein above or below.

In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment but may do. Furthermore, the features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some, but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the invention and, as such, is meant to be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as “one embodiment”, “some embodiments”, “certain embodiments”, “further embodiment”, “specific exemplary embodiments”, and/or “another embodiment”, each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the invention.

Furthermore, the ranges defined throughout the specification include the end values as well, i.e. a range of 1 to 10, between 1 to 10 imply that both 1 and 10 are included in the range. For

the avoidance of doubt, the applicant shall be entitled to any equivalents according to applicable law.

As used herein, the term "about" when qualifying a value of a stated item, number, percentage, or term refers to a range of plus or minus 10 percent, 9 percent, 8 percent, 7 percent, 6 percent, 5 percent, 4 percent, 3 percent, 2 percent or 1 percent of the value of the stated item, number, percentage, or term. Preferred is a range of plus or minus 10 percent.

In case numerical ranges are used herein such as "in a concentration between 1 and 5 micromolar", the range includes not only 1 and 5 micromolar, but also any numerical value in between 1 and 5 micromolar, for example, 2, 3 and 4 micromolar. The term "in vitro" as used herein denotes outside, or external to, the animal or human body. The term "in vitro" as used herein should be understood to include "ex vivo". The term "ex vivo" typically refers to tissues or cells removed from an animal or human body and maintained or propagated outside the body, e.g., in a culture vessel. The term "in vivo" as used herein denotes inside, or internal to, the animal or human body.

Definitions:

In order for the present disclosure to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms may be set forth throughout the specification.

The term "protein" or "polypeptide" or "(poly)peptide" or "peptide" (all terms are used interchangeably, if not indicated otherwise) as used herein encompasses isolated and/or purified and/or recombinant (poly)peptides being essentially free of other host cell polypeptides. The term "peptide" as referred to herein comprises at least two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300 or even more amino acid residues where the alpha carboxyl group of one is bound to the alpha amino group of another. A post-translational modification of the protein or peptide as used and envisaged herein is the modification of a newly formed protein or peptide and may involve deletion, substitution or addition of amino acids, chemical modification of certain amino acids, for example, amidation, acetylation, phosphorylation, glycosylation, formation of pyroglutamate, oxidation/reduction of sulfa group on a methionine, or addition of similar small molecules, to certain amino acids.

The term “Homologues” as used herein refers to bacterial, fungal, plant or animal homologues of the oxidase enzyme or rubredoxin or rubredoxin reductase useful in the invention, preferably plant homologues, but also includes truncated sequences, single-stranded DNA or RNA of the coding and non-coding DNA sequence.

Sequence identity, homology or similarity is defined herein as a relationship between two or more amino acid sequences or two or more nucleic acid sequences, as determined by comparing those sequences. Usually, sequence identities or similarities are compared over the whole length of the sequences but may also be compared only for a part of the sequences aligning with each other. Preferably, the sequence identities or similarities are compared over the whole length of the sequences, herein. In the art, "identity" or "similarity" also means the degree of sequence relatedness between polypeptide sequences or nucleic acid sequences, as the case may be, as determined by the match between such sequences. Sequence alignments can be generated with a number of software tools, such as:

Needleman and Wunsch algorithm - Needleman, Saul B. & Wunsch, Christian D. (1970). "A general method applicable to the search for similarities in the amino acid sequence of two proteins". Journal of Molecular Biology 48 (3): 443-453.

This algorithm is, for example, implemented into the “NEEDLE” program, which performs a global alignment of two sequences. The NEEDLE program is contained within, for example, the European Molecular Biology Open Software Suite (EMBOSS).

EMBOSS - a collection of various programs: The European Molecular Biology Open Software Suite (EMBOSS), Trends in Genetics 16 (6), 276 (2000).

BLOSUM (BLOcks Substitution Matrix) - typically generated on the basis of alignments of conserved regions, e.g., of protein domains (Henikoff S, Henikoff JG: Amino acid substitution matrices from protein blocks. Proceedings of the National Academy of Sciences of the USA. 1992 Nov 15; 89(22): 10915-9). One out of the many BLOSUMs is “BLOSUM62”, which is often the “default” setting for many programs, when aligning protein sequences.

BLAST (Basic Local Alignment Search Tool) - consists of several individual programs (BlastP, BlastN) which are mainly used to search for similar sequence in large sequence databases. BLAST programs also create local alignments. Typically used is the “BLAST” interface provided by NCBI (National Centre for Biotechnology Information), which is the

improved version ("BLAST2"). The "original" BLAST: Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410; BLAST2: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Sequence identity as used herein is preferably the value as determined by the EMBOSS Pairwise Alignment Algorithm "Needle". In particular, the NEEDLE program from the EMBOSS package can be used (version 2.8.0 or higher, EMBOSS: The European Molecular Biology Open Software Suite - Rice, P., et al. Trends in Genetics (2000) 16: 276-277; http://emboss.bioinformatics.nl) using the NOBRIEF option ('Brief identity and similarity' to NO) which calculates the "longest- identity". The identity, homology or similarity between the two aligned sequences is calculated as follows: Number of corresponding positions in the alignment showing an identical amino acid in both sequences divided by the total length of the alignment after subtraction of the total number of gaps in the alignment. For alignment of amino acid sequences, the default parameters are: Matrix = Blosum62; Open Gap Penalty = 10.0; Gap Extension Penalty = 0.5. For alignment of nucleic acid sequences, the default parameters are: Matrix = DNAfull; Open Gap Penalty = 10.0; Gap Extension Penalty = 0.5.

Sequence identity usually is provided as "% sequence identity" or "% identity". To determine the percent-identity between two amino acid sequences in a first step a pairwise sequence alignment is generated between those two sequences, wherein the two sequences are aligned over their complete, entire or full length (i.e., a pairwise global alignment). The alignment is generated with a program or software described herein. The preferred alignment for the purpose of this invention is that alignment, from which the highest sequence identity can be determined.

The term sequence "identity" used herein refers to the percentage identity between two aligned sequences using standard NCBI BLAST parameters (http://blast.ncbi.nlm.nih.gov).

The nucleic acids (or polynucleotides) of the invention comprises nucleic acid sequences which encode the fusion proteins of the invention. The nucleic acid sequences encoding the fusion proteins of the invention are preferably recombinant and/or isolated and/or purified nucleic acid sequences. The nucleic acid sequences which encode the fusion proteins of the

invention can be produced and isolated using known molecular-biological standard techniques, the sequence information and organisms provided herein.

The term "nucleic acid" as used herein, includes reference to a deoxyribonucleotide or ribonucleotide polymer, i.e. a polynucleotide, in either single-or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids). A polynucleotide can be full-length or a sub-sequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are "polynucleotides" as the term is used herein. Lt will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells. Every nucleic acid sequence herein that encodes a polypeptide such as the oxidase enzyme or rubredoxin or rubredoxin reductase also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, the term "conservatively modified variants" if used, may refer to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences due to the degeneracy of the genetic code. The term "degeneracy of the genetic code" refers to the fact that a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues.

The terms “polypeptide”, “peptide” and “protein” apply also to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms “polypeptide”, “peptide” and “protein” are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulphation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. Within the context of the present application, oligomers (such as oligonucleotides, oligopeptides) are considered a species of the group of polymers. Oligomers have a relatively low number of monomeric units, in general 2-100, in particular 6-100, including, e.g., primer sequences, such as used for cloning of the oxidase enzyme or rubredoxin or rubredoxin reductase useful in the invention, in the Examples.

The term “heterologous” when used with respect to a nucleic acid (DNA or RNA) or protein of the invention refers to a nucleic acid or protein that does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins of the invention are not endogenous to the cell into which they are introduced but have been obtained from another cell or synthetically or recombinantly produced. Generally, though not necessarily, such nucleic acids encode proteins that are not normally produced by the cell in which the DNA is expressed. A gene that is endogenous to a particular host cell but has been modified from its natural form, though, for example, the use of DNA shuffling, is also called heterologous. The term “heterologous” also includes non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the term “heterologous” may refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position and/or a number within the host cell nucleic acid in which the segment is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

A “homologous” DNA sequence of the invention is a DNA sequence that is naturally associated with a host cell into which it is introduced. Any nucleic acid or protein that one of skill in the art would recognize as heterologous or foreign to the cell in which it is expressed is herein encompassed by the term heterologous nucleic acid or protein.

The terms “modified”, “modification”, “mutated”, or “mutation”, as used herein regarding proteins or polypeptides compared to another protein or polypeptide apply mutatis mutandis to nucleotide or nucleic acid sequences. The mentioned terms are used to indicate that the modified nucleotide or nucleic acid sequences encoding the protein or polypeptide has at least one difference in the nucleotide or nucleic acid sequence compared to the nucleotide or nucleic acid sequence of the protein or polypeptide with which it is compared. The terms are used irrespective of whether the modified or mutated protein actually has been obtained by mutagenesis of nucleic acids encoding these amino acids or modification of the polypeptide or protein, or in another manner, e.g. using artificial gene-synthesis methodology. Mutagenesis is a well-known method in the art, and includes, for example, site-directed mutagenesis by means of PCR or via oligonucleotide- mediated mutagenesis, as described in Sambrook, J., and Russell, D.W. Molecular Cloning: A Laboratory Manual.3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (2001). The term “modified”, “modification”, “mutated”, or “mutation” as used herein regarding genes is used to indicate that at least one nucleotide in the nucleotide sequence of that gene or a regulatory sequence thereof, is different from the nucleotide sequence that it is compared. A modification or mutation may in a particular be a replacement of a nucleotide by a different one, a deletion of a nucleotide or an insertion of a nucleotide.

The terms “modified fHbp/ modified fHbp fusion protein/ recombinant protein/ chimera/ chimeric protein/ chimeric molecule/ chimeric antigen/ fusion protein/ clone” are interchangeably used throughout the specification and refers to proteins created through the joining of two or more genes which originally coded for separate or same proteins resulting in a polypeptide comprising a combination of sequences from different gene products or sources. Translation of this recombinant/ chimeric/ fusion gene results in a single polypeptide with functional properties derived from each of the original proteins along with few additional characteristics.

The term “tagged protein” used herein refers to proteins having specific peptide sequence(s) (also known as tags) grafted genetically. The tags serve various purposes and can be added to either end of the target protein are either C-terminus or N-terminus specific or are both C-terminus and N-terminus specific. Some tags are also inserted at sites within the protein of interest; they are known as internal tags.

Affinity tags are appended to proteins so that they can be purified from their crude biological source using an affinity technique. Affinity tags include chitin binding protein (CBP), maltose binding protein (MBP), Strep-tag and glutathione-S-transferase (GST). The poly(His) tag is a widely used protein tag, which binds to matrices bearing immobilized metal ions.

The term "peptide loop" used herein is intended to refer to a single chain polypeptide sequence anchored at both ends (e.g. anchored to a scaffold such as fHbp). The term "loop" does not infer or require any particular secondary structure adopted by the polypeptide.

The term "exogenous" used herein in the context of "exogenous peptide loop" is understood to mean that the peptide loop is derived from a different source relative to the fHbp protein (i.e. it is not fHbp or a fragment thereof). However, it may be from the same organism as the fHbp. For example, a modified fHbp may include an *N. meningitidis* fHbp modified with (exogenous) peptide loop(s) derived from *N. meningitidis* PorA.

The term "isolated", when applied to the modified fHbp of the present invention means a protein: (i) encoded by nucleic acids using recombinant DNA methods or a viral vector; or (ii) synthesized by, for example, chemical synthetic methods; or (iii) separated from biological materials, and then purified. An isolated polypeptide of the invention includes a protein expressed from a nucleotide sequence encoding the protein, or from a recombinant vector containing a nucleotide sequence encoding the protein.

The term "immunogenic" or "antigen" used herein refers to a molecule that is capable of eliciting an immune response in a human or animal body. The immune response may be protective.

The term "protective" used herein means prevention of a disease, a reduced risk of disease infection, transmission and/or progression, reduced severity of disease, a cure of a condition or disease, an alleviation of symptoms, or a reduction in severity of a disease or disease symptoms.

The term "prophylaxis" used herein means prevention of or protective treatment for a disease. The prophylaxis may include a reduced risk of infection, transmission and/or progression, or reduced severity of disease.

The term "treatment" used herein means a cure of a condition or disease, an alleviation of symptoms, or a reduction in severity of a disease or disease symptoms.

The terms "freeze-drying/ lyophilize/ lyophilization" refers to the process by which a suspension/solution is frozen, after which the water is removed by sublimation at low pressure.

The term "sublimation" refers to a change in the physical properties of a composition, wherein the composition changes directly from a solid state to a gaseous state without becoming a liquid.

The present disclosure envisages an efficient platform process for manufacturing an effective vaccine against *Neisseria meningitidis* that meets multiple criteria including improved immunogenicity, safety and affordability, in particular an improved formulation showing low viscosity, devoid of aggregation; showing long-term stability across wide temperature ranges.

According to an aspect of the present invention, a modified factor H binding protein (fHbp) is provided. In an embodiment, the modified factor H binding protein (fHbp) is a fusion protein and comprises a wild type fHbp variant and at least one exogenous peptide loop(s).

In one embodiment, the fHbp is meningococcal fHbp. In another embodiment, the fHbp is gonococcal fHbp.

In an embodiment the modified factor H binding protein (fHbp) has an amino acid sequence with at least 75% identity with any one of sequences of SEQ ID NOs 6 to 10.

In an embodiment, the modified factor H binding protein (fHbp) includes an amino acid sequence with at least 75% identity with any one of sequences of SEQ ID NOs 6 to 10.

The skilled person will understand that one, two, three or four or more amino acid substitutions, deletions or additions may be made to the modified fHbp of the invention herein without substantially removing its immunogenic function or affecting stability. Substitutions may be to similar amino acid residues, for example having similar MW, charge, hydrophobicity or moieties, or synthetic analogues. Such modifications are envisaged as part of the invention.

In one embodiment the modified fHbp may have at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with any one of the modified fHbp sequence described herein.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with any one of sequences of SEQ ID Nos 6 to 10.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0% identity with sequence of SEQ ID No 6. In a preferred embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 6.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0% identity with sequence of SEQ ID No 7. In a preferred embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 7.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0% identity with sequence of SEQ ID No 8. In a preferred embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 8.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0% identity with sequence of SEQ ID No 9. In a preferred embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 9.

In one embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0% identity with sequence of SEQ ID No 10. In a preferred embodiment, the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 10.

In accordance with the embodiments of the present invention, the at least one exogenous peptide loop(s) is immunogenic and is derived from a bacterial membrane protein.

In an embodiment, the fHbp variant is selected from v1, v2 and v3, and is modified with at least one PorA loop inserted into a β-turn region in fHbp. In one embodiment, the fHbp may comprise fHbp v1. In another embodiment, the fHbp may comprise fHbp v2. In another embodiment, the fHbp may comprise fHbp v3.

In an embodiment, the variant of the wild type fHbp comprises a wild type meningococcal orthologue of fHbp. For example, a variant of fHbp may comprise Ghfp, the Gonococcal homologue of fHbp. Ghfp is non-functional and closely related to v3 fHbps (>95% aa identity, dissociation constant KD > 100 μM with factor H).

In another embodiment, the PorA loop is selected from VR1, and VR2. In one embodiment, the PorA loop is VR1. In another embodiment, the PorA loop is VR2.

In an embodiment, the modified factor H binding protein is modified to reduced factor H binding activity. In a preferred embodiment, the modified fHbp has >80% decreased binding to human factor H (fH), as compared to wild type fHbp.

According to another aspect of the invention, there is provided a nucleic acid encoding essentially or at least the modified fHbp according to the invention herein.

In an embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with any one of sequences of SEQ ID NO. 1 to 5.

In one embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0% identity with sequence of SEQ ID No 1. In a preferred embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 1.

In one embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0% identity with sequence of SEQ ID No 2. In a preferred embodiment, the present invention is directed to a

nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 2.

In one embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0% identity with sequence of SEQ ID No 3. In a preferred embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 3.

In one embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0% identity with sequence of SEQ ID No 4. In a preferred embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 4.

In one embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0% identity with sequence of SEQ ID No 5. In a preferred embodiment, the present invention is directed to a nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75.0%, or 80.0%, or 85.0%, 90.0%, 95.0%, 95.0%, 98.0%, 99.0%, or 99.5% identity with sequence of SEQ ID No 5.

In an embodiment, there is provided a nucleic acid sequence encoding the modified fHbp of the present invention. In an embodiment, the nucleic acid is a vector, such as a viral vector.

According to another aspect, the present invention is directed to an immunogenic composition comprising at least one modified fHbp as described herein.

In an embodiment, the present invention is directed to an immunogenic composition comprising at least one modified fHbp as disclosed herein or encoded by the nucleic acid sequence encoding the modified fHbp as disclosed herein.

In an embodiment, there is provided an immunogenic composition comprising at least one modified fHbp of the present invention.

In an embodiment, the present invention is directed to an immunogenic composition comprising at least one modified fHbp of the present invention, wherein the modified fHbp includes

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10, or combinations thereof, or
- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5, or combinations thereof.

In an embodiment, the present invention is directed to an immunogenic composition comprising at least one modified fHbp of the present invention, wherein the modified fHbp includes

- fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1), or
- fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2), or
- fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3), or
- fHbpV1.1:PorA307-311/exP1.4 (of SEQ ID NO. 9 or encoded by SEQ ID NO. 4), or
- fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5), or combinations thereof.

In another embodiment, the immunogenic composition comprises two or more different modified fHbp.

In still another embodiment, the immunogenic composition comprises three or more different modified fHbp.

In yet another embodiment, the immunogenic composition comprises four different modified fHbp.

In an embodiment, the immunogenic composition comprises a pharmaceutically acceptable carrier. In a further embodiment, the immunogenic composition comprises an adjuvant. In another embodiment, the immunogenic composition further comprises at least one other prophylactically or therapeutically active molecule.

The at least one other prophylactically or therapeutically active molecule comprises:

- a monovalent protein: capsule polysaccharide vaccine; or

- a conjugate vaccine, wherein antigen(s) comprising the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the conjugate vaccine.

The modified fHbp, the nucleic acid or the composition of the present invention may be used as a medicament, or in treatment or prevention of a pathogenic infection or colonization of a subject.

The present invention further envisages an immunogenic composition comprising a combination of the modified fHbp, a nucleic acid, and at least one other prophylactically or therapeutically active molecule.

In an embodiment, the at least one other prophylactically or therapeutically active molecule comprises a protein: capsule polysaccharide conjugate vaccine.

In an embodiment, the protein: capsule polysaccharide vaccine comprises any of serogroup C or A capsule with bacterial toxoids, bi-valent vaccines (with serogroup C and A capsular polysaccharide conjugated to bacterial toxoids), quadrivalent- (serogroups A, C, Y, W polysaccharides conjugated to bacterial toxoids) or pentavalent- (serogroups A, C, Y, W, X polysaccharides conjugated to bacterial toxoid) conjugate vaccines.

In another embodiment, the at least one other prophylactically or therapeutically active molecule comprises a conjugate vaccine, wherein antigen(s) comprising the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the conjugate vaccine, optionally wherein the conjugate vaccine comprises any of serogroup capsular polysaccharides selected from A, C, Y, W, or X strains, or combinations thereof.

In an embodiment, the factor H binding protein (fHbp) is used as an epitope display scaffold.

fHbp found on the surface of *Neisseria meningitidis* is a 27 kDa lipoprotein that consists of two beta barrels (an N terminal barrel and a C terminal barrel) joined by a short amino acid linker. While charged carbohydrates on the surface of the vascular endothelium engage fH, charged amino acids in the fHbp bind to fH at nanomolar affinities at the same site of this complement regulator. Based on differences in the nucleotide and predicted amino acid sequences, fHbps from different Neisseria meningitidis strains have been categorised using several schemes. Factor H Binding Protein (fHbp, also referred to in the art as lipoprotein 2086 (Fletcher et al (2004) Infect Immun 72:2088-2100), Genome-derived Neisserial antigen

(GNA) 1870 (Masignani et al. (2003) J Exp Med 197:789-99) or "741") is a surface-exposed lipoprotein expressed in the N. meningitidis bacterium. Based on differences in the nucleotide and predicted amino acid sequences, fHbps from different Neisseria meningitidis strains have been categorised using several schemes. These include two subfamilies (A and B) (Murphy E, et al. (2009) The Journal of Infectious Diseases 200: 379- 389) or three variant groups (V1, V2, and V3) (Masignani V, et al. (2003) The Journal of Experimental Medicine 197: 789-799), with subfamily A corresponding to V2 and V3, and subfamily B to V1 (which is the most abundant).

fHbps belonging to the same variant group share over 85% amino acid similarity, and only 60-70% similarity between the three variant groups. fHbp is also an antigen that elicits serum bactericidal antibody responses in immunised individuals and is a key component of investigational vaccines for the prevention of meningococcal, in particular serogroup B, disease that are currently being evaluated in clinical trials. However, immunisation with a protein belonging to one variant fHbp family generates a variant specific response, with no cross-reactivity to other variant groups. So, a single fHbp does not provide universal protection against meningococcal disease, hence immunisation with representatives from each of the three variants, V1, V2 and V3, is necessary for a broad-based vaccine.

Meningococcal outer membrane vesicle (OMV) is PorA, an integral outer membrane protein (OMP) in the meningococcus. However, the sequence of this protein is diverse, and the prevalence of particular variants differs by geographic region. Variants of PorA are identified by sequences in the variable-regions (VR) of the protein, which are located in the surface-exposed peptide loops of the protein and are the target of immune responses. PorA has seven extracellular peptide loops; the fourth loop is variable region 2 (VR2) and is the target of most serum bactericidal activity (SBA) generated by PorA following natural infection and after immunisation with OMVs. SBA is a known correlate of protection against meningococcal disease. Despite sequence diversity, around 70 % of UK isolates are covered by vaccines containing six PorA proteins (http://pubmlst.org/neisseria/PorA/).

It has been shown herein that immunogenic peptides, such as those from PorA, can be introduced into factor H binding protein (fHbp), which acts as a molecular scaffold. The peptides that are introduced into fHbp are presented to the immune system and are able to elicit protective responses such as SBA. Advantageously, the fHbp molecule provides an ideal molecular scaffold for stable inclusion of peptide loops for the display of epitopes,

particularly for epitopes that are difficult to stabilise and display in their native conformation, for example loops from integral OMPs such as PorA. In particular, many OMPs, such as PorA, are difficult to express because of the insolubility of their membrane spanning domains. PorA has a 16-beta stranded barrel structure with the surface-exposed loops between strands 1 and 2 (loop 1), strands 7 and 8 (loop 4), strands 9 and 10 (loop 5) and strands 1 1 and 12 (loop 7) demonstrated to be the most effective antigens. fHbp contains two beta barrels, therefore the peptide loop sequences from OMPs can be inserted into the tips of the loops between betα- strands of fHbp to present the extra-cellular loop fragments from integral OMPs, in their native conformations for immunisation. Therefore, the modified fHbp scaffold molecule of the invention may be used as a prophylactic or a therapeutic vaccine directed to Nm or the gonococcus in which a single protein presents key epitopes from two different antigens.

In one embodiment, the modified fHbp is a fusion protein, such as a recombinant fusion protein. In another embodiment, the modified fHbp is an isolated modified fHbp molecule. In still another embodiment, the modified fHbp molecule of the invention is included as a single protein in multi-valent vaccine. In yet another embodiment, the modified fHbp is included in an OMV vaccine.

In embodiments where more than one exogenous peptide loop is inserted into fHbp, or variants thereof, the exogenous peptide loops are same, e.g. the same sequence, or substantially similar. For example, some epitopes such as PorA epitopes, may not elicit sufficient functional responses when displayed singly on fHbp. In this instance, the present invention may be used to provide the same epitope at multiple sites on the same modified fHbp molecule, thereby enhancing the immunogenic recognition of the epitope. Alternatively, the exogenous peptide loops are different relative to each other. For example, where the exogenous peptide loops are derived from a single protein, such as PorA, the different exogenous peptide loops are from distinct regions of the protein, such as PorA. In one embodiment, the different exogenous peptide loops are derived from overlapping and distinct regions of the protein, such as PorA. In embodiments where more than one exogenous peptide loop is inserted into fHbp, or variants thereof, the exogenous peptide loops are derived from different species or strains. For example, when a multivalent vaccine is desired for multiple different antigens including different organisms.

In another aspect of the invention, an effective vaccine formulation is developed comprising at least one recombinant protein/ modified fHbp fusion protein, an adjuvant and one or more pharmaceutically acceptable excipient.

In an embodiment, vaccine formulation comprising at least one recombinant protein/ modified fHbp fusion protein further additionally comprises an antigen selected from antigen peptide subvariants fHbp 3.45, fHbp 1.55, fHbp-fHbp-fHbp or any other fHbp fusion protein, fHbp-cholera toxin, multiple Por A fused to single fHbp, recombinant N. meningitidis group B NHBA fusion protein, recombinant N. meningitidis group B NadA protein, Recombinant Neisseria meningitidis group B fHbp fusion protein or Outer membrane vesicles (OMV) from Neisseria meningitidis group B strain NZ98/254.

In one embodiment, the recombinant protein may comprise of a Transferrin Binding Protein, Neisserial Heparin Binding Protein, Neisserial Surface Protein A, PorA, meningococcal enterobactin receptor FetA, Neisserial Adhesin A, or factor H binding protein (fHbp).

Yet preferably, the recombinant protein/ modified fHbp fusion protein may comprise of a modified factor H binding protein (fHbp), comprising fHbp, or a variant thereof, to act as a molecular scaffold by modification with the addition of at least one exogenous peptide loop from a different antigen.

Yet preferably, the recombinant protein/ modified fHbp fusion protein may comprise of a modified factor H binding protein (fHbp), comprising fHbp, or a variant thereof, to act as a molecular scaffold by modification with the addition of at least two exogenous peptide loops from a different antigen.

For example, the vaccine formulation may comprise of fHbp variants selected from v1 or v2 or v3. The formulation may comprise of fHbp variants selected from v1 and v2. The formulation may comprise of fHbp variants selected from v2 and v3. The formulation may comprise of fHbp variants selected from v1, v2 and v3.

The recombinant protein/ modified fHbp fusion protein is an fHbp variant selected from v1, v2 and v3, modified with at least one PorA loop comprising at least 10 amino acids inserted into a β-turn region in fHbp. In an embodiment, the PorA loop is selected from VR1, and VR2, and has >80 % decreased binding to human factor H (fH), as compared to wild type fHbp.

All the activities related to generation of recombinant modified factor H binding protein (fHbp) have been carried out at Oxford University Innovation Limited. Contents of PCT/GB2013/052215 and PCT/GB2017/052535 are incorporated herein in entirety.

In an embodiment, the vaccine formulation is developed against *Neisseria meningitidis,* wherein the vaccine formulation may comprise of one or more recombinant proteins/ modified fHbp fusion proteins derived from *Neisseria meningitidis* and pharmaceutically acceptable carrier or excipient.

In one embodiment, the recombinant protein/ modified fHbp fusion protein is derived from *Neisseria meningitidis serogroup A, B, C, H, I, K, L, 29E, W135, X, Y and Z.*

In one of preferred embodiments, the recombinant protein/ modified fHbp fusion protein is derived from *Neisseria meningitidis serogroup* B.

In one embodiment, the vaccine formulation may comprise of a one or more different variants of modified factor H binding protein (fHbp) as an antigen selected from the group comprising of:

Table-3: Variants of modified factor H binding protein (fHbp)

Sr. No.	fHbp-PorA chimeric protein variants	SEQ ID
1	fHbpV3.45 M5:PorA316-320/exP1.14	1 (NA) 6 (AA)
2	fHbpV2.19 M6:PorA316-320/exP1.4	2 (NA) 7 (AA)
3	fHbpV1.14:PorA307-311/exP1.9	3 (NA) 8 (AA)
4	fHbpV1.1:PorA307-311/exP1.4	4 (NA) 9 (AA)
5	fHbpV1.1:PorA307-311/exP1.9	5 (NA) 10 (AA)

Table-3a: Additional Variants of modified factor H binding protein (fHbp) that may be used

Sr. No.	fHbp-PorA chimeric protein variants
1	fHbp V2.19 M6 PorA $^{190/exP1.4}$
2	fHbp V2.19 M6 PorA $^{190/exP1.9}$
3	fHbp V2.19 M6 PorA $^{316-320/exP1.9}$
4	fHbp V2.19 M6 PorA $^{316-320/exP1.14}$
5	fHbp V2.19 M6 PorA $^{316-320/exP\ 1.15-11}$

6	fHbp V3.45 M5 PorA $^{190/exP1.14}$
7	fHbp V3.45 M5 PorA $^{316-320/exP1.4}$
8	fHbp V3.45 M5 PorA $^{316-320/exP1.9}$
9	fHbp V3.45 M5 PorA $^{316-320/exP\ 1.15-11}$
10	fHbp V3.45 M5 PorA $^{190/exP\ 1.15-11}$
11	fHbp V1.14 PorA $^{183/exP1.4}$
12	fHbp V1.14 PorA $^{183/exP1.9}$
13	fHbp V1.14 PorA $^{307-311/exP1.4}$
14	fHbp V1.14 PorA $^{307-311/exP1.14}$
15	fHbp V1.14 PorA $^{307-311/exP\ 1.15-11}$
16	fHbp V1.1 PorA $^{183/exP1.4}$
17	fHbp V1.1 PorA $^{183/exP1.9}$
18	fHbp V1.1 PorA $^{307-311/exP1.14}$
19	fHbp V1.1 PorA $^{307-311/exP\ 1.15-11}$

In an embodiment, the molecular weight of the recombinant protein/ modified fHbp fusion protein is in the range of 10 kDa to 200 kDa, preferably up to 50 kDa.

In an embodiment, the molecular weight of the recombinant protein/ modified fHbp fusion protein is in the range of 20 kDa to 40 kDa. In one embodiment, the formulation may comprise of four different variants of modified factor H binding protein (fHbp) selected from the group summarized in Table-4.

Table-4: Variants of modified factor H binding protein (fHbp)

Sr. No.	fHbp-PorA chimeric protein variants
1	fHbp V3.45 M5 PorA $^{316-320\ exP1.14}$
2	fHbp V2.19 PorA $^{316-320\ exP1.4}$
3	fHbp V1.14 PorA $^{307-311\ exP1.9}$
4	fHbp V1.1 PorA $^{307-311\ exP1.4}$
5	fHbp V1.1 PorA $^{307-311\ exP1.9}$

In one embodiment, the formulation may comprise of four different variants of modified factor H binding protein (fHbp) as an antigen selected from the Table-1.

In one embodiment, the recombinant protein/ modified fHbp fusion protein is adsorbed onto an adjuvant to improve the immunogenicity of the antigen and maximise the protection from the associated disease.

In one embodiment, the adsorption of recombinant protein/ modified fHbp fusion protein onto an adjuvant is evaluated and optimized for percent adsorption of same onto an adjuvant. In accordance with the embodiments of the present invention, adsorption buffers are used to improve adsorption to the surface, reduce aggregation and unfolding upon binding, reduced-dose efficacy of the recombinant protein/ modified fHbp fusion protein as an antigen post adsorption.

In one embodiment, the recombinant protein/ modified fHbp fusion protein is adsorbed onto an adjuvant selected from the group comprising of alum adjuvant based salt such as aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate, or immunostimulatory component based adjuvant selected from the group consisting of an oil and water emulsion (MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, GLA, 3–deacylated monophosphoryl lipid A, AS01, AS03, AF3)(Include all 3 vendors of MPLA that we are exploring including synthetic etc; MPL from Salmonella enterica serotype Minnesota Re 595) (for example, Sigma Aldrich Catalog # L6895).), IL-2, RANTES, GM- CSF, TNF-a, IFN-g, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL, 3–deacylated monophosphoryl lipid A, AS01, AS03, AF3), an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers, polymer p 1005, CRL-8300 adjuvant, muramyl dipeptide, such as an agonist of TLR1/2 (which can be a synthetic ligand) (for example, Pam3Cys), TLR2 (for example, CFA, Pam2Cys), TLR3 (for example, polyFC, poly A:U), TLR-4 agonists(for example, MPLA, Lipid A, and LPS), TLR5 (for example, flagellin), TLR7 (for example, gardiquimod, imiquimod, loxoribine, Resiquimod®), TLR7/8 (for example, R0848), TLR8 (for example, imidazoquionolines, ssPolyU, 3M-012), TLR9 (for example, ODN 1826 (type B), ODN 2216 (type A),, and/or TLR11/12 (for example, profilin), TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, Alpha-C-galactosylceramide, Chitosan, Interleukin-2, QS-21, ISCOMS, squalene mixtures (SAF-1), Quil A, cholera toxin B subunit, polyphosphazene and derivatives, mycobacterium cell wall preparations, mycolic acid

derivatives, non-ionic block copolymer surfactants, OMV, fHbp, saponin combination with sterols and lipids, TLR-agonists (MPL, CpG, poly-IC, imiquimod), dmLT, 1,25-dihydroxyvitamin D3, CAF01, poly [di (carboxylatophenoxy)- phosphazene] (PCPP) and Venezuelan equine encephalitis (VEE) replicon particles or a combination thereof. In an embodiment, the amount of the adjuvant is in the range of in the range of 0.5 mg/ml to 4.5 mg/ml.

In an embodiment, the adjuvant is aluminium hydroxide having particle size > 500 nm.

In an embodiment, the percent adsorption of the recombinant protein/ modified fHbp fusion protein on to an adjuvant is in the range of 70 % to 100 %.

In another embodiment, the percent adsorption of fHbp V3.45 M5 PorA $^{316\text{-}320\ exP1.14}$ on to an adjuvant is in the range of 80 % to 100 %.

In another embodiment, the percent adsorption of fHbp V1.14 PorA $^{307\text{-}311\ exP1.9}$ on to an adjuvant is in the range of 80 % to 90 %.

In another embodiment, the percent adsorption of fHbp V2.19 PorA $^{316\text{-}320\ exP1.4}$ on to an adjuvant is in the range of 80 % to 90 %.

In another embodiment, the percent adsorption of fHbp V1.1 PorA $^{307\text{-}311\ exP1.4}$ on to an adjuvant is in the range of 80 % to 90 %.

In another embodiment, the percent adsorption of fHbp V1.1 PorA $^{307\text{-}311\ exP1.9}$ on to an adjuvant is in the range of 70 % to 80 %.

In one embodiment, the pharmaceutically acceptable carrier or excipient may be selected from the group comprising of buffering agent, sugar, sugar alcohol or polyol, surfactants, polymers, salts, amino acids or pH modifiers, hydrolysed protein, preservative and liquid carriers.

Examples of the buffering agent selected from the group consisting of carbonate, phosphate, acetate, HEPES, Succinate, TRIS, borate, citrate, lactate, gluconate and tartrate, as well as more complex organic buffering agents including a phosphate buffering agent that contains sodium phosphate and/or potassium phosphate in a ratio selected to achieve the desired pH. In another example, the buffering agent contains Tris (hydroxymethyl) aminomethane, or "Tris", formulated to achieve the desired pH. Yet in another example, the buffering agent

could be the minimum essential medium with Hanks salts. Other buffers, such as HEPES, piperazine-N, N′-bis (PIPES), and 2-ethanesulfonic acid (MES) are also envisaged by the present disclosure. The buffer aids in stabilizing the recombinant protein/ modified fHbp fusion protein of the present disclosure. In an embodiment, the amount of the buffer is in the range of 0.1 mM to 300 mM.

Examples of the sugars as excipient selected from the group of trehalose, mannose, raffinose, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose, dextrose, fructose or a combination thereof. In an embodiment, the amount of the sugar is in the range of 5 mg/ml to 100 mg/ml.

Examples of the sugar alcohol or polyol as excipient selected from the group of mannitol, lactitol, sorbitol, glycerol, xylitol, maltitol, lactitol, erythritol, isomalt and hydrogenated starch hydrolysates or a combination thereof. In an embodiment, the amount of the sugar alcohol or polyol is in the range of 5 mg/ml to 100 mg/ml.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have HLB of at least 10, preferably at least 15, and more preferably at least 16. Examples of Surfactants as excipient may include non-ionic surfactants such as polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 85, nonylphenoxypolyethoxethanol, octylphenoxypolyethoxethanol, oxtoxynol 40, nonoxynol- 9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene- 660 hydroxystearate, polyoxyethylene- 35 ricinoleate, soy lecithin and a poloxamer - 0.001%-0.05%. copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWF AX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-l,2-ethanediyl) groups, with octoxynol-9 (Triton X-1OO, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are Tween 20. In an embodiment, the amount of the surfactant is in the range of

0.01 mg/ml to 2 mg/ml. Examples of the polymers may include dextran, carboxymethylcellulose, hyaluronic acid, cyclodextrin, etc.

Examples of the salts may include NaCl, KCl, KH_2PO_4, $Na_2HPO_4.2H_2O$, $CaC1_2$, $MgC1_2$, etc.

Examples of the aminoacids as excipient may include tricine, leucine, iso-leucine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Methionine, Proline, Serine, Threonine.

Examples of the hydrolyzed protein may include gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate and whey protein hydrolysate, serum albumin.

Examples of the preservative may include 2-phenoxyethanol, Benzethonium chloride (Phemerol), Phenol, m-cresol, Thiomersal, Formaldehyde, paraben esters (e.g. methyl-, ethyl, propyl- or butyl- paraben), benzalkonium chloride, benzyl alcohol, chlorobutanol, p-chlor-m-cresol, or benzyl alcohol or a combination thereof. A vaccine composition may include preservative for a single immunization or may include material for multiple immunizations (i.e. a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material. In an embodiment, the amount of the preservative is in the range of 1 mg/mL to 10 mg/ml.

Examples of the suitable liquid carriers may include WFI (water for injection) and saline.

In an embodiment, the vaccine formulation comprises:

- at least one at least one recombinant protein/ at least one modified fHbp
- aluminium hydroxide;
- mannitol;
- phosphate; and
- polysorbate.

In another embodiment, the vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10 or

- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5; and
- aluminium hydroxide;
- mannitol;
- phosphate; and
- polysorbate.

In accordance with the embodiments of the present invention, the vaccine formulation is formulated to be in a dose in the range of 0.1 mL to 1 mL, for example from 0.2 mL to 0.8 mL, 0.4 mL to 0.6 mL. In one embodiment, the vaccine formulation is formulated to be at a dose of 0.5 mL.

In accordance with the embodiments of the present invention, the recombinant protein/ modified fHbp fusion protein is present in the vaccine formulation in an amount in the range of 15 µg/ml to 200 µg/ml, for example from 20 µg/ml to 200 µg/ml, 25 µg/ml to 200 µg/ml, 25 µg/ml to 150 µg/ml, 30 µg/ml to 200 µg/ml, 30 µg/ml to 150 µg/ml, 35 µg/ml to 200 µg/ml, 35 µg/ml to 150 µg/ml, 40 µg/ml to 200 µg/ml, 40 µg/ml to 150 µg/ml, 45 µg/ml to 200 µg/ml, 45 µg/ml to 150 µg/ml, 50 µg/ml to 200 µg/ml, 50 to 150 µg/ml, 75 µg/ml to 200 µg/ml, 75 to 150 µg/ml, 120 µg/ml to 200 µg/ml, 12o µg/ml to 150 µg/ml.

In accordance with the embodiments of the present invention, the recombinant protein/ modified fHbp fusion protein is present in the vaccine formulation in an amount of 15 µg/ml, 20 µg/ml, 25 µg/ml, 30 µg/ml, 35 µg/ml, 40 µg/ml, 45 µg/ml, 50 µg/ml, 55 µg/ml, 60 µg/ml, 65 µg/ml, 70 µg/ml, 75 µg/ml, 80 µg/ml, 85 µg/ml, 90 µg/ml, 95 µg/ml, 100 µg/ml, 105 µg/ml, 110 µg/ml, 115 µg/ml, 120 µg/ml, 125 µg/ml, 130 µg/ml, 135 µg/ml, 140 µg/ml, 145 µg/ml, 150 µg/ml, 200 µg/ml.

In an embodiment, the vaccine formulation comprises (i) at least one recombinant protein/ modified fHbp fusion protein; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

In an embodiment, the vaccine formulation comprises (i) at least two recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5

mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

In an embodiment, the vaccine formulation comprises (i) at least three recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

In an embodiment, the vaccine formulation comprises (i) at least four recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

In an embodiment, the vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10 or
- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5

  and
  - aluminium hydroxide;
  - mannitol;
  - phosphate; and
  - polysorbate.

In an embodiment, vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10, in an amount in the range of 15 µg/ml to 150 µg/ml, or

- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5 , in an amount in the range of 15 µg/ml to 150 µg/ml; and
- aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
- mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

In an embodiment, vaccine formulation comprises:

- fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml; or
- fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml; or
- fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml; or
- fHbpV1.1:PorA307-311/exP1.4 (of SEQ ID NO. 9 or encoded by SEQ ID NO. 4) in an amount in the range of 15 µg/ml to 150 µg/ml; and
- aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
- mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

In an embodiment, vaccine formulation comprises:

- fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml;
- fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml;
- fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml;
- fHbpV1.1:PorA307-311/exP1.4 (of SEQ ID NO. 9 or encoded by SEQ ID NO. 4) in an amount in the range of 15 µg/ml to 150 µg/ml;
- aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
- mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

In an embodiment, vaccine formulation comprises:

- fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL; or
- fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL; or
- fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL; or
- fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/mL to 150 µg/mL; and
- aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
- mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

In an embodiment, vaccine formulation comprises:

- fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL;
- fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL;
- HbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL;
- fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/mL to 150 µg/mL;
- aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
- mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

In an embodiment, the vaccine formulation comprises 2-phenoxyethanol in an amount in range of 1 mg/mL to 10 mg/ml.

In accordance with the embodiments of the present invention, the vaccine formulation comprises (i) at least one fusion protein comprising stable non-functional/ non-lipidated fHbp and PorA VR2 loop, and (ii) at least one polysaccharide-protein conjugate.

In an embodiment, the vaccine formulation comprises i) at least one fusion protein comprising stable non-functional /non-lipidated fHbp and PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

In an embodiment, the vaccine formulation comprises i) at least two fusion proteins, each consisting of one fHbp variant type coupled to one PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

In an embodiment, the vaccine formulation comprises i) at least three fusion proteins, each consisting of one fHbp variant type coupled to two PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

In an embodiment, the vaccine formulation comprises i) at least four fusion proteins, each consisting of one fHbp variant type coupled to three PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

In an embodiment, the recombinant protein/ modified fHbp fusion protein of the present invention is *co-administered* with one or more vaccine selected from BEXSERO, MENVEO, MENACTRA, NIMENRIX, MenQuadFi, MENFIVE, MenAfriVac, Men AC, and Men ACHib.

In a preferred embodiment, the recombinant protein/ modified fHbp fusion protein of the present invention is *co-administered* with MENFIVE.

In an embodiment, the vaccine formulation comprises 2-phenoxyethanol in an amount in range of 1 mg/mL to 10 mg/ml.

In an embodiment, the vaccine formulation has Zeta potential in the range of -16 mV to -30 mV.

In an embodiment, the vaccine formulation has osmolality in the range of 200 mOsmol/kg to 500 mOsmol/kg.

The vaccine formulation described above is manufactured by the following broad steps, which are further explained in detail in the succeeding paragraphs:

- growing host cells comprising the expression vector in nutrient medium;
- inducing the host cells for expressing protein;
- harvesting and separating the host cells;
- lysing the harvested cells and separating host cell debris to obtain tagged proteins;
- purifying tagged proteins;
- removing tags from the tagged proteins to obtain recombinant proteins/ modified fHbp fusion protein;
- purifying the recombinant proteins/ modified fHbp fusion protein; and
- preparing vaccine formulation comprising the purified recombinant proteins/ modified fHbp fusion protein.

According to yet another aspect of the invention, the upstream bioprocess is developed and optimized to increase cell density and recombinant protein/ modified fHbp fusion protein productivity for the lead cell lines.

In one embodiment, the upstream bioprocess may comprise of batch, fed-batch, continuous or perfusion mode of cultivation for the production of recombinant protein/ modified fHbp fusion protein.

In one embodiment, the upstream bioprocess may comprise of fed-batch method comprising following steps:

(a) preparing an aqueous fermenter nutrient medium and feed solution;

(b) inoculating the fermenter nutrient medium with the Host cell line;

(c) continuous feeding with a feed solution;

(d) inducing protein expression;

(e) harvesting and cell separation.

In one embodiment, during upstream bioprocess development number of experiments were performed in order to achieve the high-productivity required by the prospective commercial process. The culture process development efforts focused on four main areas: (1) confirmation of the performance of the high per cell expression strains identified during screening and selection of clones with the improved baseline culture process; (2) development of an enhanced media composition capable of supporting the high-productivity culture performance; (3) adjustment of fermentation process parameters and in-process controls to enable high cell density and recombinant protein/ modified fHbp fusion protein expression; and (4) feeding strategies and optimization of amino acid additions to prevent stalling during transcription and translation.

In one embodiment, the aqueous fermenter nutrient medium may comprise of Undefined medium, Terrific Broth (TB) Medium, Lysogenia Broth, Luria Broth or Luria-Bertani medium, chemically defined medium, M9 Minimal Medium, Chemically Defined M9 Modified Salt Medium, 2xYT medium or Super Optimal broth with Catabolite repression (SOC) Medium or combination thereof.

Yet preferably the Composition of Luria Broth (LB Media) is summarized in Table-5.

Table-5: LB medium Composition

Sr No.	Component	Quantity (per Litre)
1	Tryptone	5-20 g
2	Yeast Extract	1-20 g
3	NaCl	5-20 g

NaCl – Sodium chloride

Yet preferably the Composition of Chemically Defined M9 Modified Salt Medium is summarized in Table-6.

Table-6: Chemically Defined M9 Modified Salt Medium Composition

Sr No.	Component	Quantity (per Litre)
1	Glucose	5-30 g
2	K_2HPO_4	5-30 g
3	KH_2PO_4	5-30 g
4	Citric Acid	0.5-10 g
5	$(NH_4)_2SO_4$	0.5-10 g
6	$MgSO_4,7H_2O$	0.5-10 g
7	TES	1-20 mL
8	Thiamine HCl	1-20 mg
9	L-Methionine	1-20 mM

K_2HPO_4 - Dipotassium hydrogen phosphate

KH_2PO_4 - Potassium dihydrogen phosphate

$(NH_4)_2SO_4$ - Ammonium sulfate

$MgSO_4,7H_2O$ - magnesium sulfate heptahydrate

TES - Trace Element Solution

14% liquid Ammonia Solution and 6% Orthophosphoric acid is used to maintain the pH during fermentation batch within a pH range of 6.8-8.2.

Yet preferably the Composition of Trace Element Solution is summarized in Table-7.

Table-7: Trace Element Solution Composition		
Sr No.	Component	Quantity (per Litre)
1	$FeSO_4,7H_2O$	2.8 g
2	$MnSO_4.H_2O$	2.68 g
3	$CoCl_2,6H_2O$	1.42 g
4	$CaCl_2,2H_2O$	1.5 g
5	$CuSO_4.5H_2O$	0.18 g
6	$ZnSO_4,7H_2O$	0.3 g
7	HCl	87 mL

Yet preferably the Composition of Terrific Broth (TB) Medium is summarized in Table-8.

Table-8: TB medium Composition		
Sr No.	Component	Quantity (per Litre)
1	Tryptone	5-30 g
2	Yeast Extract	5-30 g
3	Glycerol	1-20 g
4	10X PBS Stock Solution	1-200 mL

In one embodiment, the aqueous fermenter nutrient medium may additionally comprise of antibiotic selected from the group comprising of kanamycin, neomycin, streptomycin, tobramycin, paromomycin, amphotericin B, Ampicillin, Erythromycin, Gentamycin, Nystatin Penicillin-Streptomycin, Polymyxin B, Tetracyclin, Thiabendazole or Tylosin or a combination thereof.

In one embodiment, the Host cell line may comprise of bacterial expression host system.

More preferably, the bacterial expression host system is Escherichia coli (E. coli) and the E. coli strain is selected from the group comprising BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**, Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3) or B834(DE3).

** Denotes the presence of an additional plasmid*

Most preferably, the bacterial expression host system is Escherichia coli strain B834(DE3).

In one embodiment, the fermentation condition post inoculating the Host cell line may comprise of temperature in the range of 35°C to 39°C; pH in the range of 5.0 to 9.0; dissolved oxygen in the range of 10 to 100%; agitation in the range of 100–1800 rpm; gas flow rate 0-2 VVM (volume of gas per unit volume of liquid per minute).

In one embodiment, the inoculated fermenter nutrient medium may comprise of continuous feeding with a feed solution post depletion of carbon source.

Preferably the composition of feed solution comprising glucose is summarized in Table-9.

Table-9: Glucose Feed Composition

Sr No.	Component	Quantity/L
1	Glucose	100-1000 g
2	$MgSO_4,7H_2O$	10-50 g
3	Thiamine HCL	1-200 mg
4	Trace Element solution	5-20 mL

(Glucose/ dextrose/ dextrose monohydrate are used interchangeably in the present application)

Preferably the composition of feed solution comprising glycerol is summarized in Table-10.

Table-10: Glycerol Feed Composition		
Sr No.	Component	Quantity/L
1	Glycerol	100-1000 g
2	$MgSO_4,7H_2O$	10-50 g
3	Thiamine HCL	1-200 mg
4	Trace Element solution	5-20 mL

In an embodiment, the concentration of glycerol in the glycerol feed composition is in the range of 30 % to 80%. In one embodiment, the concentration of glycerol in the glycerol feed composition is 50%.

In one embodiment, the feeding rate of glucose feed is in the range of 0.3 to 3.0 mL/min/L of culture.

In one embodiment, the glucose feed may be replaced with glycerol feed solution and subsequently the host cell line may be induced for recombinant protein/ modified fHbp fusion protein expression.

In one embodiment, the feeding rate of glycerol feed is in the range of 0.3 to 3.0 mL/min/L of culture.

In one embodiment, the host cell line is induced for recombinant protein/ modified fHbp fusion protein expression using an inducer selected from lactose or its non-hydrolyzable analog isopropyl β-D-1-thiogalactopyranoside (IPTG).

In one embodiment, the induction parameters may comprise of inducing at OD measured at 590/600 nm in the range of 20-100. In an embodiment, the inducer is IPTG at a concentration in the range of 1mM to 10 mM. In another embodiment, the inducer is IPTG at a concentration in the range of 5 mM to 10 mM. In an embodiment, the inducer is lactose at a concentration in the range of 1 g/L to 50 g/L. In another embodiment, the inducer is lactose at a concentration in the range of 5 g/L to 50 g/L.

In an embodiment, the inducing/ induction temperature is in the range of 17°C to 37°C, and the induction hours is in the range of 3 to 24 hours.

In one embodiment, during induction number of experiments were performed to optimize induction density and length, culture temperature, culture pH, temperature shift, and glucose/glycerol feed rates.

In one embodiment, three different fed-batch methods/protocols for the production of chimeric proteins in fermenter is summarized in Table-11.

Table-11: fed-batch methods/protocols for the production of chimeric proteins in fermenter

Sr. no	Carbon Source in Fermentation Media	Carbon Source in Feed	Inducer
1	Glucose	Glucose	IPTG
2	Glycerol	Glycerol	IPTG/ Lactose
3	Glucose	Dual Feed A. Glucose feed in growth phase B. Glycerol feed in induction/ production phase	IPTG/ Lactose

In one embodiment, the culture is harvested at 3-7 hours after induction.

In one embodiment, post harvesting the cells are separated from the fermenter nutrient medium by centrifugation.

Preferably the centrifugation parameters may comprise of Relative Centrifugal Force (rcf) 6000-8000 for 30 – 60 min at 1°C to 5°C.

In one embodiment, post harvesting the resulting wet cell mass (g/L) may comprise of 150 – 350 g/L of harvest broth.

According to still another aspect of the invention, the downstream bioprocess is developed and optimized for production of recombinant protein/ modified fHbp fusion protein with high yield and high purity.

In one embodiment, the downstream bioprocess may comprise of any one of following steps:

a) Cell Lysis/Cell disruption;
b) Cell Separation and Clarification;
c) Purification of tagged protein;

d) Affinity Tag removal;
e) Protein purification;
f) Concentration/ diafiltration/ buffer exchange.

In one embodiment, during downstream bioprocess development a no of experiments were performed in order to achieve the high-yield and high purity required by the prospective commercial process.

In one embodiment, post harvesting and cell separation the cell culture is lysed or disrupted to make the intracellular product accessible.

In one embodiment, the cell lysis/cell disruption is carried out by a method selected from the group comprising of Chemical, Biological, Physical or Mechanical mode or a combination thereof. Wherein the chemical method of cell lysis may comprise of detergents, solvents, acid, or base or a combination thereof. Wherein the biological method of cell lysis may comprise of Lysozyme. Wherein the physical method of cell lysis may comprise of Freeze–thawing, Acoustic cavitation, Hydrodynamic cavitation or Osmotic shock or a combination thereof. Wherein the Mechanical mode of cell lysis may comprise of Grinding (e.g. bead mill) or High-pressure homogenization or a combination thereof.

In an embodiment, the cell lysis/ cell disruption is carried out by mechanical mode comprising high pressure homogenization.

In another embodiment, the cell lysis/ cell disruption is carried out by chemical lysis using a lysis buffer.

In yet another embodiment, the cell lysis/cell disruption is carried out by a combination of chemical lysis followed by mechanical mode comprising of high-pressure homogenization. In an embodiment, high pressure homogenization comprises pressure in a range from 1000-1500 Bar, Cycles in a range from 3-8, flow rate of 9 L/hour +/- 5%, temperature in a range from 4 °C to15 °C.

In an embodiment, the flow rate is increased as the process is scaled up.

In an embodiment, the mechanical lysis is carried out using a homogenizer.

In an embodiment, the lysis buffer comprises sodium phosphate in the range of 10-100 mM, pH 6.0-8.0, Imidazole in the range of 10-50 mM, Sodium chloride (NaCl) in the range of 100-500 mM.

Lysis buffer: A lysis buffer is a buffer solution used for the purpose of breaking open cells for use.

In one embodiment, post cell lysis/cell disruption the intracellular product is evaluated for disruption efficiency and potential product loss by measuring either or all of the following parameters comprising of total protein release, Cell viability and Particle size distribution.

In one embodiment, post cell lysis/ cell disruption the intracellular product is evaluated for recombinant protein/ modified fHbp fusion protein expression levels by a method selected from the group comprising of SDS-PAGE, Western blot, ELISA, enzyme assays.

Preferably the recombinant protein/ modified fHbp fusion protein may comprise of fHbp-PorA chimeric protein.

In one embodiment, the fHbp-PorA chimeric protein may comprise of affinity tags selected from the group comprising of peptide tag and/or a large polypeptide.

Affinity tag: When devising a project where a purified soluble active recombinant protein/ modified fHbp fusion protein is needed (as is often the case), it is invaluable to have means to (i) detect it along the expression and purification scheme, (ii) attain maximal solubility, and (iii) easily purify it from the E. coli cellular milieu. The expression of a stretch of amino acids (peptide tag) or a large polypeptide (fusion partner) in tandem with the desired protein to form a tagged fusion protein may allow these three goals to be straightforwardly reached.

Preferably the fHbp-PorA chimeric protein may comprise of small peptide tags selected from the group comprising of poly-Arg-, FLAG-, poly-His-, c-Myc-, S-, and Strep II-tags and a large polypeptide (fusion partner) selected from the group comprising of maltose-binding protein (MBP), N-utilization substance protein A (NusA), thioredoxin (Trx), glutathione S-transferase (GST), ubiquitin and SUMO.

Most preferably the fHbp-PorA chimeric protein may comprise of poly-His and MBP tag; wherein the expression vector may comprise of pET28a-His-MBP-TEV-fHbp-PorA.

In one embodiment, post cell lysis/cell disruption the cell mass is subjected to cell separation and clarification.

Preferably, the cell separation is carried out by centrifugation, wherein the intracellular product comprising of recombinant protein/ modified fHbp fusion protein is separated in the supernatant.

In an embodiment, centrifugation is carried out at 4000 – 10000 RPM for 30 - 60 min at 1°C to 5°C.

In one embodiment, post cell separation the supernatant is subjected to clarification for removal of lysate proteins.

In an embodiment, the clarification is carried out by using filters selected from the group comprising of decreasing pore sizes (e.g., 6 µ, 5 µ, 0.8 µ, 0.65µ, 0.45 µ, 0.2 µ). Suitable commercially available filters and filtration devices are well known in the art and can be selected by those of skill. Exemplary filtration devices could be made of Polypropylene or Cellulose acetate or Polyethersulfone and the commercially available filters could be Millipak (Millipore), Kleenpak (Pall) and Sartobran™ P filtration devices.

In one embodiment, post cell separation and clarification the supernatant comprising of tagged recombinant protein/ modified fHbp fusion protein is subjected to purification comprising of chromatography-based purification methods, ultrafiltration, diafiltration or combination thereof.

In an embodiment, the chromatography-based purification methods comprise Ion-exchange or affinity chromatography or a combination of both.

In another embodiment, the chromatography-based purification methods comprise affinity chromatography-based purification wherein the tagged recombinant protein/ modified fHbp fusion protein is bound to an affinity column and is further subjected to washing with increasing concentration of buffer comprising sodium phosphate + NaCl, + Imidazol. The tagged protein is eluted with elution buffer.

Preferably, the affinity column may comprise of Immobilized metal affinity column resin.

Immobilized metal affinity chromatography (IMAC) resin is a high binding-capacity resin for purifying his-tagged proteins wherein it relies on the affinity that His has for immobilized transition metals.

In accordance with the embodiments, the Immobilized metal affinity column resin comprises metal ions selected from copper, zinc, nickel, and the like.

Preferably, the Immobilized metal affinity column resin comprise of uncharged or precharged with Ni2+ resin.

In an embodiment, the uncharged form is charged with the metal ion of choice for even greater purification flexibility. In another embodiment, the Immobilized metal affinity column resin may comprise of Ni Sepharose 6 Fast Flow Column.

In an embodiment, the wash buffer comprises 20-50mM Sodium-Phosphate buffer, 50-300mM Sodium Chloride (NaCl) pH 7.4 and increased concentration of Imidazol comprises 20 – 80 mM (each 5-6 CV).

In an embodiment, the elution of the target protein is carried out using elution buffer comprising of 20-50mM Sodium-Phosphate buffer, 50-120mM Sodium Chloride (NaCl) pH 7.4 and Imidazol comprising of 100 -300 mM.

In an embodiment, post elution the elute comprising the tagged recombinant protein/ modified fHbp fusion protein is further subjected to concentration and diafiltration.

In an embodiment, concentration and diafiltration is carried out using tangential flow filtration (TFF) typically through filters with a molecular weight cut off (MWCO) ranging in between 5KDa -50KDa and 10-50mM Sodium-Phosphate buffer pH7.4.

In an embodiment, concentration and diafiltration is carried out using tangential flow filtration (TFF) typically through filters with a molecular weight cut off (MWCO) ranging in between 5 kDa to 50 kDa and 10-50mM Sodium-Phosphate buffer pH 7.4 or Tris-HCl buffer pH 8.5.

In one embodiment, the tagged recombinant protein/ modified fHbp fusion protein is subjected to TEV protease digestion for removal of affinity tag.

In an embodiment, the recombinant protein/ modified fHbp fusion protein comprising of poly-His and MBP tag is subjected to TEV protease digestion for removal of His-MBP tag,

wherein the TEV protease digestion comprises incubating the recombinant protein comprising of poly-His and MBP tag with TEV protease.

Tobacco etch virus (TEV) protease is a 27-kDa catalytic domain of the polyprotein nuclear inclusion a (NIa) in TEV, which recognizes the specific amino acid sequence ENLYFQG/S and cleaves between Q and G/S. In one embodiment, the tagged recombinant protein/ modified fHbp fusion protein is subjected to TEV protease digestion for removal of affinity tag, wherein the HIS-GST-TEV protease with at least 75% identity with amino acid SEQ ID NO. 12 or is encoded by nucleic acid sequence with at least 75.0% identity with sequence of SEQ ID NO. 11.

In an embodiment, the His-GST-TEV protease has a molecular size of ~50 kDa.

In a preferred embodiment, the tagged recombinant protein/ modified fHbp fusion protein is subjected to TEV protease digestion for removal of affinity tag, wherein the HIS-GST-TEV protease has at least 75% or 80.0%, or 85.0%, or 90.0% or 95.0% or 98.0%, or 99.0%, or 99.5% identity with amino acid SEQ ID NO. 12 or is encoded by nucleic acid sequence with at least 75.0%, or 80.0%, or 85.0%, or 90.0% or 95.0% or 98.0%, or 99.0%, or 99.5% identity with sequence of SEQ ID NO. 11.

In an embodiment, the ratio of tagged recombinant protein/ modified fHbp fusion protein: TEV protease is in the range of 5:1 to 30:1 or higher based on activity and purity of TEV protease. In an embodiment, the tagged recombinant protein/ modified fHbp fusion protein (substrate): TEV protease ratio is 20:1.

In an embodiment, the temperature of incubation is in the range of 4°C to 35°C and time of incubation is in the range of 15 to 20 hours.

In one embodiment, the TEV protease digestion is performed in presence of 10-50mM Sodium-Phosphate buffer pH 7.4, followed by addition of dithiothreitol (DTT) at a final concentration of 0.5 mM to 5 mM.

In an embodiment, the TEV protease used for tag removal/ digestion is produced by a method comprising the following steps:

- growing host cells comprising the expression vector in nutrient medium;
- inducing the host cells for expressing TEV protease;
- harvesting and separating the host cells;

- lysing the harvested cells and separating host cells to obtain TEV protease; and
- purifying TEV protease.

In an embodiment, the host cell may comprise of Escherichia coli (E. coli) and the E. coli strain may be selected from the group comprising of BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**, Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3), Rosetta™(DE3)pLysS or B834(DE3).

In an embodiment, the host cell for expression of TEV protease is E. coli Rosetta™(DE3)pLysS.

In an embodiment, the TEV protease enzyme produced by the aforementioned method includes the TEV protease having SEQ ID NO. 12 or encoded by SEQ ID NO. 11.

In an embodiment, the TEV protease is purified using the following non-limiting steps:

- lysing the harvested cells;
- separating host cell debris and collecting the supernatant;
- subjecting the supernatant to at least one wash using a wash buffer;
- eluting purified TEV protease with an elution buffer;
- concentrating the purified TEV protease.

In an embodiment, the wash buffer comprises sodium phosphate, sodium chloride and imidazole.

In an embodiment, the supernatant is subjected to at least 4 wash steps with wash buffer having pH 7.4.

In another embodiment, the supernatant is subjected to at least 2 wash steps with wash buffer having pH 8.5.

The method and parameters for the production and purification of TEV protease is similar to that of the tagged recombinant protein/ modified fHbp fusion protein described above and is explained in detail in succeeding examples.

In one embodiment, post tag removal the recombinant protein/ modified fHbp fusion protein is subjected to purification comprising of chromatography-based purification methods, ultrafiltration, diafiltration or combination thereof.

In accordance with the embodiments, the chromatography is selected from column chromatography, ion-exchange chromatography, anion exchange chromatography, cation exchange chromatography, column chromatography, flash chromatography, gel filtration/ size-exclusion/ gel-permeation (molecular sieve) chromatography, affinity chromatography, paper chromatography, thin-layer chromatography, gas chromatography, dye-ligand chromatography, hydrophobic interaction chromatography, pseudoaffinity chromatography, liquid chromatography, high-pressure liquid chromatography (HPLC), immobilized metal affinity chromatography, anion exchange chromatography, cation exchange chromatography, multimodal chromatography, multimodal anion exchange chromatography, electrostatic interaction chromatography, hydrogen bonding chromatography, reverse phase chromatography, and combinations thereof.

In an embodiment, the chromatography-based purification methods comprise Ion-exchange or affinity chromatography or a combination of both.

In another embodiment, the chromatography-based purification methods comprise Ion-exchange followed by affinity chromatography-based purification.

In still another embodiment, the Ion-exchange chromatography comprises anion exchange chromatography.

In yet another embodiment, the anion exchange chromatography comprises Strong anion exchange chromatography.

In an embodiment, the affinity column may comprise Immobilized metal affinity column resin.

Immobilized metal affinity chromatography (IMAC) resin is a high binding-capacity resin for purifying his-tagged proteins wherein it relies on the affinity that His has for immobilized transition metals.

In an embodiment, the Immobilized metal affinity column resin comprises uncharged or pre-charged with Ni2+ resin.

In an embodiment, the uncharged form is charged with the metal ion of choice for even greater purification flexibility.

In an embodiment, the Immobilized metal affinity column resin comprises Ni Sepharose 6 Fast Flow Column.

Chromatography could be multimodal anion exchange resin CaptoTM Adhere, Capto adhere ImpRes, Capto MMC ImpRes or any other mixed mode combination of chromatography including Ion exchange, Electrostatic interaction, Hydrogen bonding and Hydrophobic interaction.

In an embodiment, the concentration and diafiltration is carried out using tangential flow filtration (TFF) typically through filters with a molecular weight cut off (MWCO) ranging in between 5KDa -50KDa.

In one embodiment, the elute comprising the recombinant protein/ modified fHbp fusion protein is sterilized by (Direct flow filtration (DFF) through at least one sterilization grade filter to obtain a filtrate comprising sterilized recombinant protein/ modified fHbp fusion protein. Wherein the sterilization grade filter may be selected from the group comprising of 0.8 µ, 0.45 µ, 0.2 µ. Further, commercially available filters and filtration devices are well known in the art and can be selected by those of skill. Exemplary filtration devices could be made of Polypropylene or Cellulose acetate or Polyethersulfone or Polyvinylidene difluoride and the commercially available filters could be Millipak (Millipore), Kleenpak (Pall) and Sartobran™ P filtration devices.

In one embodiment, the recombinant protein/ modified fHbp fusion protein additionally comprises inclusion bodies (IB's).

Inclusion bodies (IBs): The buildups of protein aggregates/insoluble proteins are known as IBs. IB formation results from an unbalanced equilibrium between protein aggregation and solubilization.

In one embodiment, the recombinant protein/ modified fHbp fusion protein comprising of inclusion bodies (IB's) is subjected to:

a) Inclusion body wash and recovery;
b) Inclusion bodies (IBs) solubilization and Refolding;

In one embodiment, the recombinant protein/ modified fHbp fusion protein comprising of inclusion bodies (IB's) is subjected to Urea denaturation and Inclusion bodies solubilized in urea are bound to an affinity column and is further subjected to washing with reducing

concentration of urea and refolding buffer that promotes correct refolding. The target protein is eluted with elution buffer.

In an embodiment, denaturation is carried out using denaturation buffer composition comprising of 20-50mM Sodium-Phosphate buffer, 50-120mM Sodium Chloride (NaCl) pH 7.4 and 5-10M Urea.

In another embodiment, washing with reducing concentration of urea comprises washing with 20-50mM Sodium-Phosphate buffer, 50-120mM Sodium Chloride (NaCl) pH 7.4. In another embodiment, reducing concentration of Urea comprises 8M, 6M, 4M, 2M, 1M (each 5-6 CV).

In an embodiment, elution of the target protein is carried out using elution buffer comprising imidazole 40mM-400mM.

In an embodiment, the affinity column comprises Immobilized metal affinity column resin.

Immobilized metal affinity chromatography (IMAC) resin is a high binding-capacity resin for purifying his-tagged proteins wherein it relies on the affinity that His has for immobilized transition metals.

In an embodiment, the Immobilized metal affinity column resin comprises uncharged or pre-charged with Ni2+ resin.

In another embodiment, the uncharged form is charged with the metal ion of choice for even greater purification flexibility.

In still another embodiment, the Immobilized metal affinity column resin comprises Ni Sepharose 6 Fast Flow Column.

In one embodiment, the supernatant comprising of inclusion bodies is subjected to protein precipitation prior to urea denaturation.

In an embodiment, the protein precipitation is carried out using Ammonium sulphate.

In one embodiment, post Urea denaturation the supernatant is subjected to filtration using 0.22 µM filter.

In one embodiment, post elution the elute comprising the target recombinant protein/ modified fHbp fusion protein is further subjected to concentration and diafiltration.

In an embodiment, concentration and diafiltration is carried out using tangential flow filtration (TFF) typically through filters with a molecular weight cut off (MWCO) ranging in between 5kDa -50kDa.

In an embodiment, the concentration of the recombinant protein/ modified fHbp fusion protein is more than 0.95 mg/ ml.

In another embodiment, the glucose feed is stopped, and glycerol feed is started when OD at 590/600 nm is 20-100 and inducing the culture by adding and/or maintaining lactose at 1-50 g/L in fed batch mode.

In an embodiment, the purified chimeric/ recombinant protein/ modified fHbp fusion protein is stored at 2°C to 8°C till further use. In another embodiment, the purified chimeric/ recombinant protein/ modified fHbp fusion protein is stored at 2°C to 8°C in the presence of a stabilizer(s). In an embodiment, the stabilizer is selected from TRIS, Tween/polysorbate, non-ionic detergent, such as polyethyleneglycol lauryl ether (BRIJ35), sucrose (up to 5%.), and the like.

According to another aspect of the invention, the recombinant protein/ modified fHbp fusion protein formulation is optimized for improving immunogenicity, improving stability and maintaining stability over long-term storage of recombinant protein/ modified fHbp fusion protein antigens.

In one embodiment, the optimized vaccine formulation has low viscosity, is devoid of aggregation, and has long-term stability across wide temperature ranges. In an embodiment, the optimized vaccine formulation includes solid or liquid carriers.

In one embodiment, the vaccine formulation is fully liquid. Suitable forms of liquid preparation include solutions, suspensions, emulsions, syrups, isotonic aqueous solutions, viscous compositions and elixirs that are buffered to a selected pH.

In one embodiment, the vaccine formulation comprises polymers or other agents to control the consistency of the composition, and/or to control the release of the antigen/ secreted protein from the composition.

In one embodiment, the vaccine formulation is in the form of transdermal preparations including lotions, gels, sprays, ointments or other suitable techniques. If nasal or respiratory (mucosal) administration is desired (e.g., aerosol inhalation or insufflation), compositions can

be in a form and dispensed by a squeeze spray dispenser, pump dispenser or aerosol dispenser. Aerosols are usually under pressure by means of a hydrocarbon. Pump dispensers can preferably dispense a metered dose or a dose having a particular particle size. When in the form of solutions, suspensions and gels, in some embodiments, the immunogenic compositions contain a major amount of water (preferably purified water) in addition to the active ingredient(s).

In one embodiment, the vaccine formulation is stable at 2-8 °C from 12 to 36 months; at 25 °C from 2 to 6 months; at 37 °C from 1 week to 4 weeks, at 42 °C for 2-7 days, and at 55 °C for 2-7 days.

In one embodiment, the vaccine formulation is a lyophilized/ freeze dried formulation.

In one embodiment, the final pH of the formulation may be in the range of pH 6.0 to pH 8.0.

According to a further aspect of the invention, there is provided a modified fHbp, nucleic acid, or formulation according to the invention, for use in the treatment or prevention of a pathogenic infection or colonisation of a subject. According to a still further aspect of the invention, there is provided a method of treatment or prevention of a pathogenic infection or colonisation of a subject, comprising the administration of a modified fHbp, nucleic acid, or composition according to the invention to the subject. According to another aspect of the invention, there is provided a method of vaccination, comprising the administration of a modified fHbp, nucleic acid, or composition according to the invention to a subject.

In one embodiment, the modified fHbp is immunogenic involving administration of an immunologically effective amount of the immunogenic formulation on to a human subject via parenteral or subcutaneous or intradermal, intramuscular or intraperitoneal or intravenous administration or injectable administration or sustained release from implants or administration by eye drops or nasal or rectal or buccal or vaginal, peroral or intragastric or mucosal or perlinqual, alveolar or gingival or olfactory or respiratory mucosa administration or any other routes of immunization.

As used herein “co-administered” means that the different immunogenic compositions/ vaccines can be administered either separately or as a combination.

Where the vaccines are administered separately, they will typically be administered at different sites e.g. one vaccine to the left upper arm, and a second vaccine to the right upper

arm. Thus, two vaccines may be administered contralaterally (e.g. both arms or both legs, or a contralateral arm and leg) or ipsilaterally (e.g. the arm and leg on the same side of the body). Although the vaccines are administered separately, they are administered at substantially the same time (e.g. during the same medical consultation or visit to a healthcare professional or vaccination centre), such as within 1 hour of each other.

Rather than co-immunising separately, however, administration as a combination may be performed. Thus, co-immunisation may use a combination vaccine i.e. a single composition in which the different immunogens are admixed. Combination vaccines offer subjects the advantage of receiving a reduced number of injections, which can lead to the clinical advantage of increased compliance.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (e.g. subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular administration to the thigh or the upper arm is preferred. Injection may be via a needle (e.g. a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is about 0.5 ml.

The composition may further be provided in a 'multidose' kit, i.e., a single container containing sufficient composition for multiple immunisations. Multidoses may include a preservative, or the multidose container may have an aseptic adaptor for removal of individual doses of the composition.

The subject who is immunized is a human being, who may be any age e.g. 0-12 months old, 1 -5 years old, 5-18 years old, 18-55 years old, or more than 55 years old. Preferably, the subject who is immunized is an adolescent (e.g. 12-18 years old) or an adult (18 years or older).

Optionally, the subject is an adolescent or adult who has been immunized against N. meningitidis in childhood (e.g. before 12 years of age), and who receives a booster dose of an immunogenic composition according to the invention.

In an embodiment, the vaccine formulation of the present invention elucidates cross protection against Neisseria gonorrhea strains and Neisseria meningitidis serogroups ACWYX.

The administration may be provided in a therapeutically effective amount. A skilled person will be capable of determining an appropriate dosage and repetitions for administration.

The vaccine formulation could be formulated as single dose vials or multidose vials (2 Dose or 5 Dose or 10 Dose vials) or multidose kit or as pre-filled syringes wherein the said vaccine formulation may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination is followed by 1-3 separate doses given at subsequent time intervals after 1-3 years if needed. The dosage regimen will also, at least in part, be determined on the need of a booster dose required to confer protective immunity.

In an embodiment, the vaccine formulation is formulated for administration to a human subject elders, adolescents, adults or children less than 2 years of age or more than 2 years of age according to a one dose or two dose regimens or 3 dose regimens consisting of a first dose and/or a second dose to be administered between 3 months to 2 years after the first dose and/or a third dose to be administered between 3 months to 2 years after the second dose.

In an embodiment, the subject is a mammal, such as human.

In an embodiment, the infection is a bacterial infection. In another embodiment, the infection is meningitis, such as *Neisseria meningitidis, or Neisseria gonorrhoeae.*

According to a further aspect of the invention, there is provided a combination of the modified fHbp according to the invention and at least one other prophylactically or therapeutically active molecule.

In an embodiment, the at least one other prophylactically or therapeutically active molecule comprises a vaccine or antigen different to the modified fHbp according to the invention herein. In accordance with the embodiments of the present invention, the antigen is selected from but not limited to Diphtheria toxoid (D), Tetanus toxoid (T), Whole cell pertussis (wP), hepatitis B virus surface antigen (HBsAg), Haemophilus influenzae b PRP-Carrier protein conjugate (Hib), Haemophilus influenzae (a, c, d, e, f serotypes and the unencapsulated strains), Neisseria meningitidis A antigen(s), Neisseria meningitidis C antigen(s), Neisseria meningitidis W-135 antigen(s), Neisseria meningitidis Y antigen(s), Neisseria meningitidis X antigen(s), Streptococcus Pneumoniae antigen(s), Neisseria meningitidis B bleb or purified antigen(s), Staphylococcus aureus antigen(s), Anthrax, BCG, Hepatitis (A, C, D, E, F and G strains) antigen(s), Human papilloma virus, HIV, Salmonella typhi antigen(s), acellular pertussis, modified adenylate cyclase, Malaria Antigen (RTS,S), Measles, Mumps, Rubella,

Dengue, Zika, Ebola, Chikungunya, Japanese encephalitis, rotavirus, Diarrheal antigens, Flavivirus, smallpox, yellow fever, Shingles, and Varicella virus antigens.

In another embodiment, the present invention envisages compositions comprising antigens for immunising against other diseases or infections in addition to fHbp-PorA chimeric antigens. In accordance with the embodiments, the composition comprises the following additional antigens:

- a protein antigen from PorB, Fet A, OmpC, NHBA, NadA, meningococcal antigen 287, NspA, HmbR, NhhA, App, 936,
- a saccharide antigen from N. meningitidis serogroup A, C, W, Y and/or X,
- a saccharide antigen from Streptococcus pneumoniae,
- a diphtheria antigen, such as a diphtheria toxoid e.g. the CRM197 mutant,
- a tetanus antigen, such as a tetanus toxoid,
- an antigen from Bordetella pertussis, acellular or whole cell pertussis antigens,
- a saccharide antigen from Haemophilus influenzae B,
- polio antigen(s) such as IPV,
- measles, mumps and/or rubella antigens,
- influenza antigen(s), such as the haemagglutinin and/or neuraminidase surface proteins,
- antigen (protein or saccharide) from Streptococcus agalactiae (group B streptococcus),
- antigen (protein or saccharide) from Streptococcus pyogenes (group A streptococcus),
- antigen (protein or saccharide) from Staphylococcus aureus,
- antigen (protein or saccharide) from Salmonella Spp.

In one embodiment, the at least one other prophylactically or therapeutically active molecule comprises a monovalent capsule polysaccharide- protein conjugate vaccine. The monovalent protein capsule polysaccharide vaccine may comprise any of *Neisseria meningitidis* serogroup C or A capsule with bacterial toxoids, bi-valent vaccines (with serogroup C and A capsular polysaccharide conjugated to bacterial toxoids), quadrivalent (serogroups A, C, Y, W) or pentavalent (A, C, Y, W, X) conjugate vaccines. Alternatively, the at least one other prophylactically or therapeutically active molecule may comprise a conjugate vaccine, wherein antigen(s) comprising the fHbp scaffold bearing exogenous peptide loops (such as PorA loops) may be incorporated as the protein carrier molecule in the conjugate vaccine. The conjugate vaccine may comprise any of serogroup capsular polysaccharides from A, C, Y, W, or X strains individually or in combination.

Combination Vaccines could be selected from Hexavalent (ACWYX-B), Quadrivalent (AC-Hib-B), Trivalent (AC-B), Bivalent (A-B, X-B, C-B).

In an embodiment, the liquid N meningitidis serogroup B vaccine is reconstituted with Lyophilized ACWYX conjugate vaccine for a bed side administration.

According to embodiment, instant invention relates to a method of inducing an immune response against Neisseria meningitidis in a mammal. The method includes administering to the mammal an effective amount of an immunogenic composition including i) at least one fusion protein comprising stable non-functional /non-lipidated fHbp and PorA VR2 loop of PorA and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

An aspect of the present invention relates to a method of inducing an immune response against Neisseria meningitidis in a mammal. The method includes administering to the mammal an effective amount of an immunogenic composition including i) at least two fusion proteins, each consisting of one fHbp variant type coupled to one PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

Another aspect of the invention relates to a method of inducing an immune response against Neisseria meningitidis in a mammal. The method includes administering to the mammal an effective amount of an immunogenic composition including i) at least three fusion proteins, each consisting of one fHbp variant type coupled to two PorA VR2 loop and atleast one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup

W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

Yet another aspect of the instant invention relates to a method of inducing an immune response against Neisseria meningitidis in a mammal. The method includes administering to the mammal an effective amount of an immunogenic composition including i) at least four fusion proteins, each consisting of one fHbp variant type coupled to three PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

fHbp-PorA Chimeric antigens for use with the invention comprise an amino acid sequence: having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to Sequence as disclosed in the sequence listing of SEQ ID 1-10. The sequence listings are enclosed in the standard ST.26 format and are incorporated herein by reference.

List of Men B strains used for testing of fHbp-PorA chimeric vaccine

The fHbp isogenic strains expressing different fHbps (V1.1, V1.14, V2.19 and V3.45 in H44/76ΔfHbpΔPorA constructs were used for testing of fHbp-PorA chimeric vaccine:

A. <u>fHbp expressing strains:</u>

1. H44/76ΔfHbpΔPorA:fHbp V1.1
2. H44/76ΔfHbpΔPorA:fHbp V1.14
3. H44/76ΔfHbpΔPorA:fHbp V2.19
4. H44/76ΔfHbpΔPorA:fHbp V3.45

B. <u>PorA expressing strains:</u>

1. H44/76ΔfHbpΔPorA:PorA 1.4
2. H44/76ΔfHbpΔPorA:PorA 1.9

C. <u>Clinical isolate:</u>

M11.240413 (expressing fHbp V1.13 and PorA 1.9)

Following clinical strains of N. Meningitidis would be used for SBA at Oxford/UKHSA for testing of fHbp-PorA chimeric vaccine:

Sr. No.	Strain Isolate ID	Clonal Complex expressing fHbp and PorA	PubMLST ID
1	M08 240157	ST-32 complex (fHbp V1.1, PorA 1.4)	40818
2	M17 240832	ST-41/44 complex (fHbp V1.14, PorA 1.9)	60604
3	M17 240156	ST-213 complex (fHbp V3.45, PorA 1.15-11)	53319
4	M18 240043	ST-269 complex (fHbp V2.19, PorA 1.14)	60955

Additionally, N. meningitidis strains M15 240912, M16 240272, M15 240460 or any other appropriate strain may be used for SBA.

According to another aspect of the invention, there is provided the use of factor H binding protein (fHbp) as an epitope display scaffold. The use as an epitope display scaffold may comprise the use of a factor H binding protein (fHbp) comprising any of the modifications described herein. In addition to their potential use as vaccines, compositions or modified fHbps according to the invention may be useful as diagnostic reagents and as a measure of the immune competence of a vaccine.

The immune response elicited by the modified fHbp of the invention may affect the ability of Neisseria meningitidis (Nm) to infect a subject immunised with the modified fHbp of the invention. Preferably, the ability of Nm to infect a subject immunised with the modified fHbp of the invention is impeded or prevented. The immune response elicited may recognise and destroy Nm.

Alternatively, or additionally, the immune response elicited may impede or prevent replication of Nm. Alternatively, or additionally, the immune response elicited may impede or prevent Nm causing disease in the human or non-human animal.

The foregoing description of the specific embodiments fully reveals the general nature of the embodiments herein that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It

is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. Therefore, while the embodiments herein have been described in terms of preferred embodiments, those skilled in the art will recognize that the embodiments herein can be practiced with modification within the spirit and scope of the embodiments as described herein.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The use of the expression "one or more" or "at least one" suggests the use of one or more elements or ingredients or quantities, as the use may be in the embodiment of the invention to achieve one or more of the desired objects or results.

Any discussion of documents, acts, materials, devices, articles or the like that has been included in this specification is solely for the purpose of providing a context for the disclosure. It is not to be taken as an admission that any or all of these matters form a part of the prior art base or were common general knowledge in the field relevant to the disclosure as it existed anywhere before the priority date of this application.

The numerical values given for various physical parameters, dimensions and quantities are only approximate values and it is envisaged that the values higher than the numerical value assigned to the physical parameters, dimensions and quantities fall within the scope of the invention unless there is a statement in the specification to the contrary.

While considerable emphasis has been placed herein on the specific features of the preferred embodiment, it will be appreciated that many additional features can be added and that many changes can be made in the preferred embodiment without departing from the principles of the disclosure. These and other changes in the preferred embodiment of the disclosure will be apparent to those skilled in the art from the disclosure herein, whereby it is to be distinctly understood that the foregoing descriptive matter is to be interpreted merely as illustration of the disclosure and not as a limitation.

TECHNICAL ADVANTAGES:

1. The present invention provides an efficient platform process for manufacturing an effective vaccine against *Neisseria meningitidis* that meets multiple criterion including improved immunogenicity, safety, and affordability.
2. The present invention provides a method to develop and optimize upstream bioprocess to increase cell density and recombinant protein/ modified fHbp fusion protein productivity for the lead cell lines.
3. The present invention provides a method to develop and optimize downstream bioprocess for production of recombinant protein/ modified fHbp fusion protein with high yield and high purity.
4. The improved formulation overcomes the limitations of the prior-art and shows low viscosity, devoid of aggregation; showing long-term stability across wide temperature ranges indirectly preserving the desired characteristics of the recombinant protein/ modified fHbp fusion protein, including high stability and immunogenicity.
5. The new formulation/composition i) devoid of aggregates and particle formation, higher osmolality, optimized Zeta potential, low viscosity iii) preserves the desired physicochemical & immunogenic characteristics of the recombinant protein/ modified fHbp fusion protein stability and immunogenicity for 12 months at 2-8°C, for 6 months at 25°C, for 30 days at 40°C.
6. Chimeric antigens (ChAs) against serogroup B N. meningitidis. ChAs exploit fHbp (non-lipidated) as a molecular scaffold to present the surface exposed PorA VR2 loop, which is achieved by inserting the VR2 loop ("10-20 amino acid" PorA VR2 loops instead of a "whole PorA protein) into a β-turn region in fHbp. ChAs retain epitopes from both fHbp and PorA and are found to elicit functional immune responses against both antigens. The integration of a VR2 loop does not alter the overall architecture of fHbp and that the VR2 loop folds into a conformation recognized by a bactericidal mAb.
7. The chimeric proteins that are soluble and high yielding are stable, wherein fHbp and the PorA VR2 loop both are immunogenic.
8. The Chimeras generated are composed of the most prevalent fHbp and PorA antigens to maximize vaccine coverage as Chimera composition mirrors the prevalent fHbp and PorA antigens circulating within a given geographical area.
9. The insertion of PorA loop at a specific position in fHbp results in desired reduction in Factor H binding (reduced by at least 10 %, at least 50%; preferably >70% as compared to wild type) & having Molecular weight in the range of 20 kDa to 40 kDa, while preserving immunogenic epitopes of both fHbp & PorA (Table-12).

Table-12: fHbp-PorA chimeras		
Sr. No. of Chimera	**Description of chimera**	**MW in kDa**
Chimera 1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	20 - 40
Chimera 2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	20 - 40
Chimera 3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	20 - 40
Chimera 4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	20 - 40
Chimera 5	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	20 - 40

The present invention is illustrated in more detail by the following embodiments and combinations of embodiments which result from the corresponding dependency references and links:

I. A modified factor H binding protein (fHbp) comprising wild type fHbp variant and at least one exogenous peptide loop(s), wherein
   a. the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75% identity with any one of sequences of SEQ ID Nos 6 to 10,
   b. wherein the at least one exogenous peptide loop(s) is immunogenic,
   c. wherein the at least one exogenous peptide loop(s) is derived from a bacterial membrane protein,
   d. wherein the modified fHbp is a fusion protein,
   e. wherein the fHbp variant is selected from v1, v2 and v3, modified with at least one PorA loop comprising at least 10 amino acids inserted into a β-turn region in fHbp; and
   f. wherein the PorA loop is selected from VR1, and VR2.

II. The modified factor H binding protein as disclosed in embodiment I, wherein the modified fHbp includes a wild type fHbp variant and at least one exogenous bacterial membrane protein peptide loop(s), or wherein the modified fHbp is a fusion protein.

III. The modified factor H binding protein as disclosed in any one of previous embodiments, wherein the modified fHbp is modified to reduced factor H binding activity.

IV. A nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75% identity with any one of sequences of SEQ ID NO. 1 to 5.

V. An immunogenic composition comprising at least one modified fHbp as disclosed in any one of previous embodiments or the nucleic acid sequence encoding the modified fHbp as disclosed in embodiment IV.

VI. The immunogenic composition as disclosed in embodiment V, wherein the composition comprises two or more different modified fHbp.

VII. The immunogenic composition as disclosed in any one of the previous embodiments, wherein the composition comprises a pharmaceutically acceptable carrier.

VIII. The immunogenic composition as disclosed in any one of the previous embodiments, wherein the composition further comprises an adjuvant.

IX. The immunogenic composition as disclosed in any one of the previous embodiments, wherein the composition further comprises at least one other prophylactically or therapeutically active molecule comprising a monovalent protein: capsule polysaccharide vaccine.

X. The immunogenic composition as disclosed in any one of the previous embodiments, wherein the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the conjugate vaccine.

XI. The immunogenic composition as disclosed in any one of the previous embodiments comprising a recombinant protein/ modified fHbp fusion protein in combination with atleast one additional antigen selected from:

- a protein antigen from PorB, Fet A, OmpC, NHBA, NadA, meningococcal antigen 287, NspA, HmbR, NhhA, App, 936,
- a saccharide antigen from N. meningitidis serogroup A, C, W, Y and/or X,
- a saccharide antigen from Streptococcus pneumoniae,
- a diphtheria antigen, such as a diphtheria toxoid e.g. the CRM197 mutant,
- a tetanus antigen, such as a tetanus toxoid,
- an antigen from Bordetella pertussis, acellular or whole cell pertussis antigens,
- a saccharide antigen from Haemophilus influenzae B,
- polio antigen(s) such as IPV,
- measles, mumps and/or rubella antigens,
- influenza antigen(s), such as the haemagglutinin and/or neuraminidase surface proteins,
- antigen (protein or saccharide) from Streptococcus agalactiae (group B streptococcus),
- antigen (protein or saccharide) from Streptococcus pyogenes (group A streptococcus),
- antigen (protein or saccharide) from Staphylococcus aureus,
- antigen (protein or saccharide) from Salmonella Spp.

XII. The immunogenic composition as disclosed in any one of the previous embodiments, wherein the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the polysaccharide conjugate vaccine selected from (A, X, C, W, Y), Bivalent (A-B, X-B, C-B), Trivalent (AC-B, AC-Hib), Quadrivalent (AC-Hib-B), Pentavalent (ACWYX) or Hexavalent (ACWYX-B).

XIII. The modified fHbp as disclosed in any one of embodiments I to III, a nucleic acid as disclosed in embodiment IV, or a composition as disclosed in any one of

embodiments V to IX, for use as a medicament, or in treatment or prevention of a pathogenic infection or colonization of a subject.

XIV. A combination of the modified fHbp as disclosed in any one of embodiments I to III, a nucleic acid as disclosed in embodiment IV, or a composition as disclosed in any one of embodiments V to IX, and at least one other prophylactically or therapeutically active molecule.

XV. The combination as disclosed in embodiment XIII or IX, wherein the at least one other prophylactically or therapeutically active molecule comprises a conjugate vaccine, comprising any of serogroup capsular polysaccharides selected from A, C, Y, W, or X strains, or combinations thereof.

XVI. The combination as disclosed in embodiment XV or the composition as disclosed in embodiment IX, wherein the protein: capsule polysaccharide vaccine comprises any of serogroup C or A capsule with bacterial toxoids, bivalent vaccines (with serogroup C and A capsular polysaccharide conjugated to bacterial toxoids), quadrivalent (serogroups A, C, Y, W polysaccharides conjugated to bacterial toxoids) or pentavalent (serogroups A, C, Y, W, X polysaccharides conjugated to bacterial toxoid) conjugate vaccines.

XVII. The factor H binding protein (fHbp) as disclosed in embodiments I to III as and when used as an epitope display scaffold.

XVIII. A vaccine formulation comprising at least one recombinant protein/ modified fHbp fusion protein an adjuvant and one or more pharmaceutically acceptable excipient, wherein the recombinant protein/ modified fHbp fusion protein is at least one selected from the group consisting of Transferrin Binding Protein, Neisserial Heparin Binding Protein, Neisserial Surface Protein A, PorA, meningococcal enterobactin receptor FetA, Neisserial Adhesin A, the factor H binding protein (fHbp) as disclosed in any one of the previous embodiments or a combination thereof.

XIX. The vaccine formulation as disclosed in embodiment XVIII, wherein the recombinant protein is fHbp as disclosed in any one of the previous embodiments and wherein the

fHbp is derived from Neisseria meningitidis serogroup A, B, C, H, I, K, L, 29E, W135, X, Y and Z.

XX. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the recombinant protein is fHbp as disclosed in any one of the previous embodiments and wherein the fHbp is derived from Neisseria meningitidis serogroup B.

XXI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the recombinant protein is fHbp as disclosed in any one of the previous embodiments and wherein the fHbp has molecular weight in the range of 10 kDa to 200 kDa, preferably up to 50 kDa.

XXII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the adjuvant is selected from the group consisting of aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate, MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, GLA, 3–deacylated monophosphoryl lipid A, AS01, AS03, AF3, IL-2, RANTES, GM- CSF, TNF-a, IFN-g, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL, an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers, polymer p 1005, CRL-8300 adjuvant, muramyl dipeptide, such as an agonist of TLR1/2 TLR2, TLR3, TLR-4 agonists, TLR5, TLR7, TLR7/8, TLR8, TLR9, ODN 2216 (type A), TLR11/12, TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, Alpha-C-galactosylceramide, Chitosan, Interleukin-2, QS-21, squalene, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, mycobacterium cell wall preparations, mycolic acid derivatives, non-ionic block copolymer surfactants, OMV, fHbp, saponin combination with sterols and lipids, dmLT, 1,25-dihydroxyvitamin D3, CAF01, poly [di (carboxylatophenoxy)- phosphazene] (PCPP) and Venezuelan equine encephalitis (VEE) replicon particles or a combination thereof.

XXIII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the adjuvant is aluminium hydroxide having particle size >500 nm.

XXIV. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the one or more pharmaceutically acceptable excipient is

a. a buffering agent selected from carbonate, phosphate, acetate, HEPES, Succinate, TRIS, borate, citrate, lactate, gluconate, tartrate, or a combination thereof;
b. a sugar selected from trehalose, mannose, raffinose, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose, dextrose, fructose, or a combination thereof;
c. a sugar alcohol or polyol selected from mannitol, lactitol, sorbitol, glycerol, xylitol, maltitol, lactitol, erythritol, isomalt and hydrogenated starch hydrolysates or a combination thereof;
d. a surfactant selected from polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 85, nonylphenoxypolyethoxethanol, octylphenoxypolyethoxethanol, oxtoxynol 40, nonoxynol- 9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene- 660 hydroxystearate, polyoxyethylene- 35 ricinoleate, soy lecithin, a poloxamer, copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), octoxynols, phospholipids, nonylphenol ethoxylates, polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols, sorbitan esters or a combination thereof;
e. a polymer selected from dextran, carboxymethylcellulose, hyaluronic acid, cyclodextrin or a combination thereof;
f. a salt selected from NaCl, KCl, KH_2PO_4, $Na_2HPO_4.2H_2O$, $CaC1_2$, $MgC1_2$, or a combination thereof;
g. an amino acid selected from tricine, leucine, iso-leucine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Methionine, Proline, Serine, Threonine, or a combination thereof;

h. a hydrolysed protein selected from gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate, whey protein hydrolysate, serum albumin or a combination thereof;

i. a preservative selected from phenoxyethanol, Benzethonium chloride (Phemerol), Phenol, m-cresol, Thiomersal, Formaldehyde, paraben esters, benzalkonium chloride, benzyl alcohol, chlorobutanol, p-chlor-m-cresol, benzyl alcohol or a combination thereof; and

j. a liquid carrier selected from water for injection (WFI) or saline.

XXV. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the vaccine formulation comprises:

- at least one at least one recombinant protein/ at least one modified fHbp as disclosed in any one of previous embodiments;
- aluminium hydroxide;
- mannitol;
- phosphate; and
- polysorbate.

XXVI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10; or
- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5; and
- aluminium hydroxide;
- mannitol;
- phosphate; and
- polysorbate.

XXVII. The vaccine formulation as disclosed in any one of the previous embodiments, comprising (i) at least one recombinant protein/ modified fHbp fusion protein; (ii)

aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

XXVIII. The vaccine formulation as disclosed in any one of the previous embodiments, comprising (i) at least two recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

XXIX. The vaccine formulation as disclosed in any one of the previous embodiments, comprising (i) at least three recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

XXX. The vaccine formulation as disclosed in any one of the previous embodiments, comprising (i) at least four recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

XXXI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10; or

- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5; and
- aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
- mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

XXXII. The vaccine formulation as disclosed in any one of the previous embodiments, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml; or
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml; or
c. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml; or
d. fHbpV1.1:PorA307-311/exP1.4 (of SEQ ID NO. 9 or encoded by SEQ ID NO. 4); in an amount in the range of 15 µg/ml to 150 µg/ml; and
e. aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
f. mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

XXXIII. The vaccine formulation as disclosed in any one of the previous embodiments, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml;
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml;
c. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml;

d. fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4 or 9) in an amount in the range of 15 µg/ml to 150 µg/ml;
e. aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
f. mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

XXXIV. The vaccine formulation as disclosed in any one of the previous embodiments, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL; or
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL; or
c. fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL; or
d. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/mL to 150 µg/mL; and
e. aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
f. mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

XXXV. The vaccine formulation as disclosed in any one of the previous embodiments, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL;
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL;
c. fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL;
d. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/mL to 150 µg/mL;
e. aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
f. mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;

g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

XXXVI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the formulation comprises 2-phenoxyethanol in an amount in range of 1 mg/mL to 10 mg/ml.

XXXVII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the vaccine composition is stable at 2-8°C, 25°C and 40°C for over a period of six months.

XXXVIII. The vaccine formulation as disclosed in any one of the previous embodiments having Zeta potential in the range of -16 mV to -30 mV; osmolality in the range of 200 mOsmol/kg to 500 mOsmol/kg.

XXXIX. The vaccine formulation as disclosed in any one of the previous embodiments, further comprising one or more antigen selected from Diphtheria toxoid (D), Tetanus toxoid (T), Whole cell pertussis (wP), hepatitis B virus surface antigen (HBsAg), Haemophilus influenzae b PRP-Carrier protein conjugate (Hib), Haemophilus influenzae (a, c, d, e, f serotypes and the unencapsulated strains), Neisseria meningitidis A antigen(s), Neisseria meningitidis C antigen(s), Neisseria meningitidis W-135 antigen(s), Neisseria meningitidis Y antigen(s), Neisseria meningitidis X antigen(s), Streptococcus Pneumoniae antigen(s), Neisseria meningitidis B bleb or purified antigen(s), Staphylococcus aureus antigen(s), Anthrax, BCG, Hepatitis (A, C, D, E, F and G strains) antigen(s), Human papilloma virus, HIV, Salmonella typhi antigen(s), acellular pertussis, modified adenylate cyclase, Malaria Antigen (RTS,S), Measles, Mumps, Rubella, Dengue, Zika, Ebola, Chikungunya, Japanese encephalitis, rotavirus, Diarrheal antigens, Flavivirus, smallpox, yellow fever, Shingles, Varicella virus antigens, and combinations thereof.

XL. The vaccine formulation as disclosed in any one of the previous embodiments comprising (i) at least one fusion protein comprising stable non-functional/ non-lipidated fHbp and PorA VR2 loop, and (ii) at least one polysaccharide-protein conjugate.

XLI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the recombinant protein/ modified fHbp fusion protein is co-administered with one or more vaccine selected from BEXSERO, MENVEO, MENACTRA, NIMENRIX, MenQuadFi, MENFIVE, MenAfriVac, Men AC, and Men ACHib.

XLII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the recombinant protein/ modified fHbp fusion protein is co-administered with MENFIVE.

XLIII. The vaccine formulation as disclosed in any one of the previous embodiments comprising i) at least one fusion protein comprising stable non-functional /non-lipidated fHbp and PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

XLIV. The vaccine formulation as disclosed in any one of the previous embodiments comprising i) at least two fusion proteins, each consisting of one fHbp variant type coupled to one PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

XLV. The vaccine formulation as disclosed in any one of the previous embodiments comprising i) at least three fusion proteins, each consisting of one fHbp variant type coupled to two PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii)

tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

XLVI. The vaccine formulation as disclosed in any one of the previous embodiments comprising i) at least four fusion proteins, each consisting of one fHbp variant type coupled to three PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

XLVII. The vaccine formulation as disclosed in any one of the previous embodiments for use in the treatment or prevention of infection and/or disease caused by Neisseria meningitidis serogroup B.

XLVIII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the vaccine formulation elucidates cross protection against Neisseria gonorrhea strains and Neisseria meningitidis serogroups ACWYX.

XLIX. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the percent adsorption of the recombinant protein/ modified fHbp fusion protein on to an adjuvant is in the range of 70% to 100%.

L. The vaccine formulation as disclosed in any one the previous embodiments, wherein percent adsorption of fHbp V3.45 M5 PorA 316-320 exP1.14 on to an adjuvant is in the range of 80 % to 100 %

LI. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the percent adsorption of fHbp V1.14 PorA 307-311 exP1.9 on to an adjuvant is in the range of 80 % to 90 %.

LII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the percent adsorption of fHbp V2.19 PorA 316-320 exP1.4 on to an adjuvant is in the range of 80 % to 90 %.

LIII. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the percent adsorption of fHbp V1.1 PorA 307-311 exP1.4 on to an adjuvant is in the range of 80 % to 90 %.

LIV. The vaccine formulation as disclosed in any one of the previous embodiments, wherein the percent adsorption of fHbp V1.1 PorA 307-311 exP1.9 on to an adjuvant is in the range of 70 % to 80 %.

LV. A method for manufacturing a vaccine formulation as disclosed in any one of the preceding embodiments, the method comprising the following steps:

a. growing host cells comprising the expression vector in nutrient medium;
b. inducing the host cells for expressing protein;
c. harvesting and separating the host cells;
d. lysing the harvested cells and separating host cell debris to obtain tagged proteins;
e. purifying the tagged proteins;
f. removing tags from the tagged proteins to obtain recombinant proteins/ modified fHbp fusion protein;
g. purifying the recombinant proteins/ modified fHbp fusion protein; and
h. preparing the vaccine formulation comprising the purified recombinant proteins/ modified fHbp fusion protein.

LVI. The method as disclosed in any one of the previous embodiments, wherein the host cell is a bacterial expression host system.

LVII. The method as disclosed in any one of the previous embodiments, wherein the bacterial expression host system is an Escherichia coli strain selected from the group consisting of BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**, Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3) or B834(DE3).

LVIII. The method as disclosed in any one of the previous embodiments, wherein the nutrient medium is selected from Undefined medium, Terrific Broth (TB) Medium, Lysogenia Broth, Luria Broth or Luria-Bertani medium, chemically defined medium, M9 Minimal Medium, Chemically Defined M9 Modified Salt Medium, 2xYT medium or Super Optimal broth with Catabolite repression (SOC) Medium and combinations thereof.

LIX. The method as disclosed in any one of the previous embodiments, wherein the concentration of L-methionine during growth of host cells in step (a) is maintained in the range of 1 mM to 10 mM, wherein the fermentation is in fed-batch mode.

LX. The method as disclosed in any one of the previous embodiments, wherein the host cells are grown at a temperature in the range of 35°C to 39°C; pH in the range of 5.0 to 9.0; dissolved oxygen in the range of 10 to 100%; agitation in the range of 100–1800 rpm; gas flow rate in the range of 0-2 volume of gas per unit volume of liquid per minute (VVM).

LXI. The method as disclosed in any one of the previous embodiments, wherein the host cells are induced using an inducer selected from lactose and its non-hydrolyzable analog isopropyl β-D-1-thiogalactopyranoside (IPTG).

LXII. The method as disclosed in any one of the previous embodiments, wherein the concentration of lactose is in the range of 1 g/L to 50 g/L and the concentration of IPTG is in the range of 1 mM to 10 mM.

LXIII. The method as disclosed in any one of the previous embodiments, wherein the host cells are lysed using a method selected from chemical mode, biological mode, physical mode, mechanical mode, and a combination thereof.

LXIV. The method as disclosed in any one of the previous embodiments, wherein the host cells are lysed using a combination of chemical and mechanical mode.

LXV. The method as disclosed in any one of the previous embodiments, wherein the host cells are lysed using a lysis buffer having a pH in the range of 7 to 9, followed by mechanical lysis at a pressure in the range of 1000-1500 Bar for 3 to 8 cycles.

LXVI. The method as disclosed in any one of the previous embodiments, wherein the mechanical lysis is carried out using a homogenizer.

LXVII. The method as disclosed in any one of the previous embodiments, wherein the tagged protein in step (e) is purified using chromatography step, followed by concentration and diafiltration.

LXVIII. The method as disclosed in any one of the previous embodiments, wherein the tags are removed using TEV protease having a protein: TEV protease ratio in the range of 5:1 to 30:1.

LXIX. The method as disclosed in any one of the previous embodiments, wherein the recombinant protein/ modified fHbp fusion protein in step (g) is purified using chromatography step, followed by concentration and diafiltration.

LXX. The method as disclosed in any one of the previous embodiments, wherein the chromatography is selected from column chromatography, ion-exchange chromatography, anion exchange chromatography, cation exchange chromatography, column chromatography, flash chromatography, gel filtration/ size-exclusion/ gel-permeation (molecular sieve) chromatography, affinity chromatography, paper chromatography, thin-layer chromatography, gas chromatography, dye-ligand chromatography, hydrophobic interaction chromatography, pseudoaffinity chromatography, liquid chromatography, high-pressure liquid chromatography

(HPLC), immobilized metal affinity chromatography, anion exchange chromatography, cation exchange chromatography, multimodal chromatography, multimodal anion exchange chromatography, electrostatic interaction chromatography, hydrogen bonding chromatography, reverse phase chromatography, and combinations thereof.

LXXI. The method as disclosed in any one of the previous embodiments, wherein the tagged proteins are expressed as inclusion bodies (IB) and are purified by urea unfolding of protein and on column refolding of the protein.

LXXII. The method as disclosed in any one of the previous embodiments, wherein the vaccine formulation is prepared by adsorbing individual recombinant protein/ modified fHbp fusion protein on to an adjuvant, followed by addition to an excipient mixture comprising a sugar alcohol, a buffering agent, a stabilizer, and a liquid carrier.

LXXIII. The method as disclosed in any one of the previous embodiments, wherein the excipient mixture comprises a preservative.

LXXIV. The method as disclosed in any one of the previous embodiments, wherein TEV protease is produced by a method comprising the following steps:

a. growing host cells comprising the expression vector in nutrient medium;
b. inducing the host cells for expressing TEV protease;
c. harvesting and separating the host cells;
d. lysing the harvested and separating host cells to obtain TEV protease; and
e. purifying the TEV protease; and
f. concentrating/ diafiltration and storage of purified TEV protease.

LXXV. The method as disclosed in any one of the previous embodiments, wherein the host cell for expressing TEV protease is an Escherichia coli strain selected from the group consisting of BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**,

Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3), Rosetta™(DE3)pLysS or B834(DE3).

LXXVI. The method as disclosed in any one of the previous embodiments, wherein the glucose feed is stopped, and glycerol feed is started when OD at 590/600 nm is 20-100 and inducing the culture by adding and/or maintaining lactose at 1-50 g/L in fed batch mode.

LXXVII. A method for inducing an immune response against Neisseria meningitidis serogroup B strain in an individual by administering to the individual a vaccine formulation as disclosed in any one of the previous embodiments, wherein the step of administration induces an immune response against the Neisseria meningitidis serogroup B strain.

EXAMPLES

The foregoing description of the embodiments has been provided for purposes of illustration and not intended to limit the scope of the present disclosure. Individual components of a particular embodiment are generally not limited to that particular embodiment, but are interchangeable. Such variations are not to be regarded as a departure from the present disclosure, and all such modifications are considered to be within the scope of the present disclosure.

The present invention is further described in light of the following examples which are set forth for illustration purpose only and not to be construed for limiting the scope of the disclosure.

The method for manufacturing the vaccine formulation in accordance with the present disclosure comprises the following broad steps, which are further explained in detail in the succeeding paragraphs:

- growing host cells comprising the expression vector in nutrient medium;
- inducing the host cells for expressing protein;
- harvesting and separating the host cells;
- lysing the harvested cells and separating host cell debris to obtain tagged proteins;
- purifying the tagged proteins;
- removing tags from the tagged proteins to obtain recombinant proteins/ modified fHbp fusion protein;

- purifying the recombinant proteins/ modified fHbp fusion proteins;
- preparing vaccine formulation comprising the purified recombinant proteins/ modified fHbp fusion protein.

Example-1: Expression of tagged fHbp proteins and TEV protease

Source of biological resources used in the present disclosure:

1) **Host Cell:** E. coli B834(DE3) was used for the expression of recombinant protein; Cat. No. D48175, Sigma Aldrich (Transferred to Serum Institute of India Pvt. Ltd. from Oxford University)
2) **Plasmid*:** pET-28a(+) DNA; Cat. No. D48556, EMD Millipore (Transferred to Serum Institute of India Pvt. Ltd. from Oxford University)
3) **Host Cell:** Rosetta™(DE3)pLysS Competent Cells was used for the expression of TEV protease; Cat. No. D49062, Sigma Aldrich
4) **Plasmid***: pET-28a(+) DNA; Cat. No. D48556, EMD Millipore (Transferred to Serum Institute of India Pvt. Ltd. from Oxford University)

(*The genes of interest are cloned/ inserted into the backbone of pET-28a(+) plasmid)

1A: Expression of tagged fHbp proteins

33 clone constructs expressing fHbp-PorA chimeric protein were received from University of Oxford. Out of the 33 clones received, 24 clones were screened for protein expression, details of which are provided in Table-3. Of the 24 clones screened, 5 clones were finalized based on the molecular and biochemical characterization, for the vaccine formulation as enlisted in Table-13. The sequence listing of recombinant protein/ modified fHbp fusion protein and TEV protease is provided in Table-13a.

Table-13: Clones for vaccine formulation

Sr. No	Clones finalized for vaccine formulation
1	fHbp V3.45 M5: PorA $^{316\text{-}320/exP1.14}$
2	fHbp V2.19 M6: PorA $^{316\text{-}320/exP1.4}$
3	fHbp V1.14: PorA $^{307\text{-}311/exP1.9}$
4	fHbp V1.1: PorA $^{307\text{-}311/exP1.4}$
5	fHbp V1.1:PorA $^{307\text{-}311/exP1.9}$

Table-13a: Sequence listing of recombinant protein/ modified fHbp fusion protein and TEV protease

Seq Id	Name	Base pair length	Type

No			
1	HIS-MBP-TEV-fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1)	1968	Nucleic acid
2	HIS-MBP-TEV-fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2)	1956	Nucleic acid
3	HIS-MBP-TEV-fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3)	1950	Nucleic acid
4	HIS-MBP-TEV-fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4)	1959	Nucleic acid
5	HIS-MBP-TEV-fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5)	1950	Nucleic acid
6	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 6)	270	Amino acid
7	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 7)	266	Amino acid
8	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 8)	264	Amino acid
9	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 9)	267	Amino acid
10	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 10)	264	Amino acid
11	HIS GST TEV Protease	1395	Nucleic acid
12	HIS GST TEV Protease	464	Amino acid

The expression vector used for the expression of the fHbp tagged proteins is pET28a-His-MBP-TEV-fHbp-PorA. Provided below in Table-14 and Figures 1-5 are the details of the vector map and nucleotide sequence.

Table-14: Vector map and nucleotide sequence of the clones used for expression of fHbp tagged proteins

Clone	Vector map	Nucleotide sequence
fHbp V3.45 M5: PorA $^{316\text{-}320/exP1.14}$	Figure-1	SEQ ID NO. 1
fHbp V2.19 M6: PorA $^{316\text{-}320/exP1.4}$	Figure-2	SEQ ID NO. 2
fHbp V1.14: PorA $^{307\text{-}311/exP1.9}$	Figure-3	SEQ ID NO. 3
fHbp V1.1: PorA $^{307\text{-}311/exP1.4}$	Figure-4	SEQ ID NO. 4
fHbp V1.1:PorA $^{307\text{-}311/exP1.9}$	Figure-5	SEQ ID NO. 5

fHbp tagged proteins production was carried out at 10 L scale fermentation batch. The details of media used are provided in Tables 15 to 19.

Stock Solutions for media and feed preparation:

1) Base Solution: 14% Ammonia Solution

2) Acid Solution: 6% Ortho Phosphoric Acid

3) Antifoam Solution: 10% Antifoam (STRUKTOL® J 673 A)

Table-15: Composition of Trace Element Solution (TES)

Sr. No.	Component	Quantity (per Litre)
1	Ferrous Sulphate Heptahydrate	2.8 g
2	Manganese Sulphate Monohydrate	2.68 g
3	Cobalt Chloride Hexahydrate	1.42 g
4	Calcium Chloride Dihydrate	1.5 g
5	Copper Sulphate Pentahydrate	0.18 g

6	Zinc Sulphate Heptahydrate	0.3 g
7	Hydrochloric Acid	87 mL

Table-16: Composition of Seed Media		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	10 g
2	Dipotassium Hydrogen Phosphate	15 g
3	Potassium Dihydrogen Phosphate	7.5 g
4	Citric Acid	2.0 g
5	Ammonium Sulphate	2.5 g
6	Magnesium Sulphate Heptahydrate	1.4 g
7	Trace Element Solution	10 mL
8	Thiamine Hydrochloride	10 mg
9	L-Methionine	750 mg
Final pH of the Seed Media is 7.0 ± 0.2		

Table-17: Composition of Fermentation Media		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	20 g
2	Glycerol	2 g
3	Dipotassium Hydrogen Phosphate	15 g
4	Potassium Dihydrogen Phosphate	7.5 g
5	Citric Acid	2.0 g
6	Ammonium Sulphate	2.5 g
7	Magnesium Sulphate Heptahydrate	1.4 g
8	Trace Element Solution	10 mL
9	Thiamine Hydrochloride	10 mg
10	L-Methionine	750 mg*
Final pH of the Fermentation Media is 7.0 ± 0.2 * 750 mg/ L of L-methionine corresponds to 5 mM of L-methionine		

Details of Feed Solutions:

Methionine Stock Solution: 45mg/mL L-Methionine Solution

Table-18: Glucose Feed		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	500 g
2	Magnesium Sulphate Heptahydrate	24.6 g
3	Thiamine Hydrochloride	70 mg
4	Trace Element Solution	15 mL

Table-19: Glycerol Feed		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Glycerol	500 g
2	Magnesium Sulphate Heptahydrate	24.6 g
3	Thiamine Hydrochloride	70 mg
4	Trace Element Solution	15 mL

Inducer: Lactose Monohydrate Solution

The process for seed development for 10 L scale fermentation batch for the production of tagged fHbp protein is provided in Figure-7. The process for the production of tagged fHbp protein at 0 L scale fermenter is provided in Figure-8.

Each of the tagged fHbp protein were individually grown as per the process provided in Figures 7 to 8.

During purification of tagged protein from cell lysate, protein precipitation for clones fHbp V3.45 M5: PorA $^{316\text{-}320/exP1.14}$ and fHbp V2.19 M6: PorA $^{316\text{-}320/exP1.4}$ was encountered. To address this issue, during fermentation process, at the time of induction, the temperature set point was shifted from 37°C to 33°C.

The impact of the temperature shift is given below:

Clone fHbp V2.19 M6: PorA $^{316\text{-}320/exP1.4}$:

- Low temperature induction (33°C) led to the major portion of tagged fHbp protein expressed in soluble form.
- Lowering the temperature by 4°C resolved the issue of protein precipitation during downstream processing.

Clone fHbp V3.45 M5: PorA $^{316\text{-}320/exP1.14}$:

- Low temperature induction (33°C) led to the major portion of tagged fHbp protein expression in soluble form.
- Although the issue is resolved to some extent, the problem still persists during downstream processing.

The growth profile for the tagged fHbp proteins at 10 L scale fermentation batch along with the SDS PAGE gel images are provided in Figures 9a to 9h. Figures 9a and 9b illustrate the

growth profile at 10 L scale fermentation batch and SDS PAGE gel images, respectively for fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6); Figures 9c and 9d illustrate the growth profile at 10 L scale fermentation batch and SDS PAGE gel images, respectively for fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7); Figures 9e and 9f illustrate the growth profile at 10 L scale fermentation batch and SDS PAGE gel images, respectively for fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8); and Figures 9g and 9h illustrate the growth profile at 10 L scale fermentation batch and SDS PAGE gel images, respectively for fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9).

It is seen from SDS PAGE analysis (Figure 9b, 9d, 9f, and 9h) that major portion of all the recombinant tagged fHbp proteins (60 kDa to 80 kDa) were expressed in soluble form.

1B: Expression of TEV protease

The expression vector used for the expression of the TEV protease is His-Gst-TEV protease (pET28a) (Kan/Chloramphenicol). Provided below in Table-20 and Figure are the detail of the vector map and nucleotide sequence.

Table-20: Vector map and nucleotide sequence of the clones used for expression of TEV protease

Clone	Vector map	Nucleotide sequence
HIS-GST-TEV protease	Figure-6	SEQ ID NO. 11

TEV protease production was carried out at 10L scale fermentation batch. The details of media used are provided in Tables 21 to 24.

Stock solutions for media and feed preparation:

1) Base Solution: 14% Ammonia Solution

2) Acid Solution: 6% Ortho Phosphoric Acid

3) Antifoam Solution: 10% Antifoam (STRUKTOL® J 673 A)

Table-21: Composition of Trace Element Solution (TES)

Sr. No.	Component	Quantity (per Litre)
1	Ferrous Sulphate Heptahydrate	2.8 g
2	Manganese Sulphate Monohydrate	2.68 g
3	Cobalt Chloride Hexahydrate	1.42 g
4	Calcium Chloride Dihydrate	1.5 g
5	Copper Sulphate Pentahydrate	0.18 g

6	Zinc Sulphate Heptahydrate	0.3 g
7	Hydrochloric Acid	87 mL

Table-22: Composition of Seed Media		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	10 g
2	Dipotassium Hydrogen Phosphate	15 g
3	Potassium Dihydrogen Phosphate	7.5 g
4	Citric Acid	2.0 g
5	Ammonium Sulphate	2.5 g
6	Magnesium Sulphate Heptahydrate	1.4 g
7	Trace Element Solution	10 mL
8	Thiamine Hydrochloride	10 mg
9	L-Methionine	750 mg
10	Kanamycin Sulfate	50 mg
Final pH of the Seed Media is 7.0 ± 0.2		

Table-23: Composition of Fermentation Media		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	20 g
2	Dipotassium Hydrogen Phosphate	15 g
3	Potassium Dihydrogen Phosphate	7.5 g
4	Citric Acid	2.0 g
5	Ammonium Sulphate	2.5 g
6	Magnesium Sulphate Heptahydrate	1.4 g
7	Trace Element Solution	10 mL
8	Thiamine Hydrochloride	10 mg
9	L-Methionine	750 mg*
10	Kanamycin Sulfate	50 mg
Final pH of the Fermentation Media is 7.0 ± 0.2 * 750 mg/ L of L-methionine corresponds to 5 mM of L-methionine		

Details of Feed Solutions –

L-Methionine Stock Solution: 45mg/mL L-Methionine Solution

Table-24: Glucose Feed		
Sr. No.	**Component**	**Quantity (per Litre)**
1	Dextrose Monohydrate	500 g
2	Magnesium Sulphate Heptahydrate	24.6 g
3	Thiamine Hydrochloride	70 mg

4	Trace Element Solution	15 mL
4	Kanamycin Sulfate	50 mg

Inducer - IPTG Solution

The process for seed development for 10 L scale fermentation batch for the production of TEV protease is provided in Figure-10. The for the production of TEV protease at 10 L scale fermenter is provided in Figure-11.

During purification of a TEV protease from cell lysate, protein precipitation was encountered. To address this issue, during fermentation process, at the time of induction, the temperature set point was shifted from 37°C to 20°C, i.e., the culture was induced by IPTG for 12-16 hours at 20°C.

The impact of the temperature shift is given below:

- Low temperature induction (20°C) led to the major portion of TEV protein expressed in soluble form.
- Low temperature induction (20°C) resolved the issue of protein precipitation during downstream processing.
- As culture was induced at 20°C, induction time increased to 12-16 hours.

The growth profile for TEV protease at 10 L scale fermentation batch along with the SDS PAGE gel images are provided in Figure-12a and Figure-12b, respectively. It is seen from SDS PAGE analysis (Figure-12b) that major portion of TEV protease (50 kDa) was expressed in soluble form.

Example-2: Purification of tagged fHbp proteins and TEV protease

The harvested cells obtained in Example-1 are subjected to purification using the following general steps in any order:

- cell lysis by chemical and mechanical means;
- chromatographic separation;
- one or more washing steps;
- elution of the protein;
- concentration and diafiltration of the protein
- storage of the protein till further use.

In case the tagged proteins are expressed in inclusion bodies (IB), additional steps of ammonium sulphate precipitation, urea unfolding of protein and on column refolding of the protein were carried out.

The purification steps are elucidated in detail in the following sections. Studies for optimizing various parameters, such as, concentration of chimeric proteins, excipients, and the like were carried out, which are explained in detail below.

Optimization studies:

Optimization of parameters such as mechanical lysis of cells, and TEV protease cleavage were conducted.

i) Homogenizer optimization:

Harvested *E. coli* cell pellet (for expression of tagged fHbp protein and TEV protease) was dissolved in lysis buffer (25 mM Na-Phosphate buffer+100mM NaCl + 20mM Imidazol, pH 7.4) and the cell were lysed with Panda Homogenizer at 1000-1200 Bar for 6 cycles. 1st cycle was run without pressure and next 5 cycles were run at 1000-1200 Bar pressure.

After each cycle lysate was collected and optical density determined using spectrophotometer at 600 nm. Results are displayed in Table-25.

Table-25: Optical density of lysate after each cycle of homogenization

Sr. No.	Homogenization cycle	Optical density at 600 nm	Dilution
1	Resuspension	0.930	01:20
2	No pressure	0.898	01:20
3	Cycle 1 (1000-1200 Bar)	0.181	01:20
4	Cycle 2 (1000-1200 Bar)	0.042	01:20
5	Cycle 3 (1000-1200 Bar)	0.024	01:20
6	Cycle 4 (1000-1200 Bar)	0.019	01:20
7	Cycle 5 (1000-1200 Bar)	0.019	01:20

Conclusion- Optical density (OD) of the cell resuspension reduced after applying pressure in the 1st cycle only. Further reduction in OD is observed till cycle 3. Cycle 4 and 5 do not show significant difference in OD.

Considering resuspension sample having 100% viable cells and 0% lysis, percentage of lysis was calculated for all homogenization cycles and the results obtained are illustrated in Table-26 and Figure-13.

Table-26: % lysis for homogenization cycles	
Homogenization cycle	**% Lysis**
Resuspension	0
No Pressure	3.4409
Cycle 1	80.5376
Cycle 2	95.48387
Cycle 3	97.41935
Cycle 4	97.95699
Cycle 5	97.95699

Conclusion- It is seen from Table-26 and Figure-13 that ~80 % lysis was achieved in cycle 1 and further reduction to ~98 % by the end of cycle 3. So at least/ minimum 3 cycles of homogenization with 1000-1200 Bar pressure is needed for efficient lysis of *E. coli* cells.

It is to be noted that in the present method chemical lysis along with mechanical lysis is used for disrupting the cells. Lysis buffer having higher osmolarity was used for chemical lysis and cell homogenizer was used for mechanical lysis.

Homogenizer was used for large scale (Industrial/ production scale; Model wise capacity-10L/Hr to thousands of L/Hr) cell disruption and to provide uniformity in lysis, whereas sonication was used for small scale (below 100ml) cell disruption.

ii) TEV protease cleavage process:

– **Enzyme: Substrate concentration optimization:**

His-MBP tagged protein (FHBP V1.1: PorA $^{307\text{-}311/\text{exP1.4}}$) was used to optimize TEV protease Enzyme: Substrate concentration (substrate here is the tagged fHbp proteins). For 1 part of TEV protease enzyme, different parts of substrate 5, 10, 20, 30 and 40 was studied. Incubation carried out at 30°C for 1 hour with gentle mixing and sample collected. Further same reactions were incubated at 30°C for 18 hours with gentle mixing as per Example-2C. TEV protease cleavage reactions were loaded on SDS-PAGE and the results obtained are illustrated in Figures 14a and 14b.

Further, Densitometric analysis of Figure-14b was carried out using Biorad gel documentation system, wherein intensity of band was measured, and percentage of product formed was calculated considering 100% cleavage at 1:5 ratio. The results obtained are illustrated in Table-27 and Figure-15.

Table-27: Densitometric analysis of TEV protease cleavage reactions

Enzyme: Substrate ratio	Incubation Time	Band Intensity	% product formed
1:5	18hr	1,73,37,698	100
1:10	18hr	1,69,08,753	97.5
1:20	18hr	1,64,12,903	94.6
1:30	18hr	1,40,72,853	81.1
1:40	18hr	1,32,40,769	76.3

Conclusion**:** It is seen from Table-27 and Figure-15 that after 1 hour of incubation using 1:5 and 1:10 Enzyme: Substrate ratios, higher degree of cleavage was obtained. However, after incubation for 18 hours, saturation for 1:5, 1:10 and 1:20 was observed, i.e., ~95% and above product formation. Uncleaved substrate was observed at higher Enzyme: Substrate ratios of 1:30 and 1:40, product formation ~ 81% and 76%, respectively suggesting that at these higher Enzyme: Substrate ratios, longer time duration for complete cleavage might be required. Thus, for TEV cleavage, Enzyme: Substrate ratio of 1:20 or lower Enzyme: Substrate ratios incubated for 18 hours are found to be optimum.

Similar findings were also observed for the remaining tagged fHbp proteins.

– **Temperature optimization for TEV protease cleavage experiment and results-**

His-MBP tagged protein (FHBP V1.1: PorA 307-311/exP1.4) was used to optimize TEV protease cleavage at various temperatures 5°C to 50°C. For 1 part of TEV protease enzyme 20 parts of substrate was used. Incubation was carried out at various temperatures 5°C to 50°C for 1 hour with gentle mixing and sample was collected. TEV protease cleavage reactions at various temperatures loaded on SDS-PAGE as illustrated in Figure-16. Further, Densitometric analysis of Figure-16 was carried out using Biorad gel documentation system, wherein intensity of band measured, and fold change of product formed was calculated considering that at initial temperature of 5°C, product formed intensity has value 1. Results obtained are displayed in Table-28 and Figure-17.

Table-28: Densitometric analysis of TEV cleavage products			
Temperature	Incubation Time	Band Intensity	Fold change
5°C	1hr	27,69,543	1
10°C	1hr	31,71,273	1.14
20°C	1hr	45,21,956	1.63
30°C	1hr	69,56,920	2.51
40°C	1hr	55,25,733	1.99
50°C	1hr	20,03,602	0.72

Conclusion**:** It is seen from Table-28 and Figure-17 that at 0 hour, no cleavage was observed. After 1 hour product/ chimera formed was higher at 30°C, which is 2.5 fold than that at 5°C. Hence, optimum temperature for TEV protease cleavage was found to be around 30°C.

Example-2A: Purification of TEV protease

The TEV protease harvested in Example-1B was purified using the process as per Figure-18, wherein lysis buffer had pH of 7.4.

The isoelectric point (pI) of a protein is defined as the pH at which the net charge of a protein molecule is zero. At solution pH that is above the pI, the surface of the protein is predominantly negatively charged, and therefore like-charged molecules will exhibit repulsive forces. Likewise, at a solution pH that is below the pI, the surface of the protein is predominantly positively charged, and repulsion between proteins occurs. However, at the pI, the negative and positive charges are balanced, reducing repulsive electrostatic forces, and the attraction forces predominate, causing aggregation and precipitation.

HIS-GST-TEV protease has a pI of 7.8 and hence, working pH of 7.4 did not result in the desired purity. Accordingly, the working pH was increased to 8.5 resulting in enhanced purity of HIS-GST-TEV protease as per the process illustrated in Figure-19.

Figures 20a and 20b illustrate the purification profiles of TEV protease at pH 8.5 and pH 7.4, respectively. Lane marked with Star symbol (★) represents the final elution fraction of TEV protease. Purity of TEV protease produced using the process with pH 8.5 is higher as compared to TEV protease produced using process with pH 7.4.

Example-2B: Purification of tagged fHbp proteins

The tagged fHbp proteins harvested in Example-1A were individually purified as per Figure-21.

The tagged fHbp proteins are soluble at the working pH used for the purification and the above mentioned process (Figure-21) results in tagged fHbp proteins with desired purity. The purification process illustrated in Figure-21 was used for purification of fHbpV3.45 M5:PorA316-320/exP1.14, fHbpV2.19 M6:PorA316-320/exP1.4, fHbpV1.14:PorA307-311/exP1.9, fHbpV1.1:PorA307-311/exP1.9, and fHbpV1.1:PorA307-311/exP1.4.

However, it is possible that during scaling up, the tagged fHbp proteins expressed at high rate may have solubility issues due to protein aggregation into inclusion bodies (IB). The IBs are formed as a result of the aggregation of partially folded and misfolded protein molecules.

To avoid such solubility issues and recover the expressed proteins from the IBs, an alternate process (Figure-22) involving urea may be used.

Inclusion bodies are known to have protein molecules in native-like conformation with some inclusion bodies having significant biological activity.

In the alternative method (Figure-22), the tagged proteins expressed as IBs were solubilized with 8M urea and subsequently bound to Ni-Sepharose 6FF column for on column refolding and purification.

Urea was removed from column by reducing its concentration from 8 M to 1 M in first step. Subsequent washing with 25 mM Na-Phosphate buffer+100mM NaCl, pH 7.4 for 5-6 column volumes removes urea completely from the column and from recombinant protein/ modified fHbp fusion protein which is bound to the column.

Example-2C: Process for tag removal from purified tagged fHbp proteins

The His-MBP tag from purified proteins (tagged fHbp proteins) was removed using TEV protease enzyme (SEQ ID NO. 12 or encoded by SEQ ID NO. 11) as per the process in Figure-23.

After removal of the tags from the fHbp protein, the chimeric protein obtained in the digestion mixture was further purified by 2-step chromatography using ion exchange chromatography (Figure-24) followed by affinity chromatography (Figure-25).

The characterization of the chimeric proteins produced using the method of the present disclosure is provided in Table-29.

Table-29: Physicochemical parameters of the chimeric proteins

Tests	Methods	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)
Identity	PorA binding ELISA	Positive response was observed against Anti-POR-A antibody				
	fHbp binding ELISA	> 85% decreased fH binding compared to wild type fHbp	> 85% decreased fH binding compared to wild type fHbp	> 90% decreased fH binding compared to wild type fHbp	> 90% decreased fH binding compared to wild type fHbp	> 90% decreased fH binding compared to wild type fHbp
Quantity	Total protein concentration by BCA*	1.18 mg/mL	1.15 mg/mL	1.6 mg/mL	0.99 mg/mL	1.8 mg/mL
Purity (Product related)	SE-HPLC	98.3% monomer	98.4% Monomer	98.9% Monomer	96.0% Monomer	97.0% Monomer
	SDS-PAGE (reduced condition)	29.5 kDa	29.5 kDa	29.7 kDa	28.3 kDa	27.9 kDa
Purity (Process related)	HCP	97.9 ng/mg	54.7 ng/mg	8.3 ng/mg	13.3 ng/mg	31.4 ng/mg
	HCD	0.14 ng/mg	0.09 ng/mg	0.09 ng/mg	0.13 ng/mg	0.15 ng/mg
	Ni leachate by AES	<0.080 ppm	<0.080 ppm	<0.080 ppm	0.119 ppm	0.096 ppm
Micro	Bacterial Endotoxin Test	605.2 EU/mg	140.9 EU/mg	31.3 EU/mg	50.5 EU/mg	27.8 EU/mg

fH= human factor H

*BCA method is explained in detail in Example-8.

The fHbp specifically binds to human factor H (fH), which down-regulates complement activation and enhances resistance to bactericidal activity. The modification(s) introduced into fHbp antigen results in decreased fH binding and can increase protective antibody responses.

It is seen from Table-29, that the recombinant fHbp have > 85% decreased fH binding compared to wild type fHbp, which will aid in improving the immunogenicity of the vaccine formulations containing these recombinant fHbp.

Previously multiple steps of chromatography were used for purification of the digestion mixture including Metal affinity chromatography, Cation exchange, Anion exchange and Gel filtration chromatography (GFC). Whereas the present method used 2 chromatography steps (Anion exchange and Metal affinity chromatography) post TEV protease mediated tag removal. The chimeric proteins produced using 2 chromatography steps of the present disclosure exhibited similar characteristics and purity profiles as that of produced using multi step chromatography.

Example-3: Formulations comprising recombinant proteins/ modified fHbp fusion protein/ chimeric proteins

Different formulations of recombinant proteins/ modified fHbp fusion protein were prepared and evaluated to obtain formulation(s) with optimum physicochemical characteristics, stability, and immunogenicity.

i) Adjuvant optimization:

To enhance immunogenic responses and physicochemical properties, formulation development started with adsorbing proteins on different adjuvants like Aluminium hydroxide (Alhydrogel), Aluminium phosphate (Adjuphos), double mutant heat-labile toxin (dmLT), etc. These formulations were evaluated on the basis of different physicochemical parameters. The summary of trials, its outcome and conclusion are provided below.

Table-30: Adjuvant Optimization		
Batch no. 290121-A		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V2.19 M6 PorA$^{316\text{-}320\ exp\ 1.4}$	100 µg/mL
2	dmLT	1 µg/mL
3	Sodium Chloride	0.9%v/v
4	Polysorbate 20	0.01% v/v
5	Phosphate buffer	10mM
6	Water for Injection	q.s.
Result	**pH**	**7.27**
	Zeta potential	**-5.07 mV**
Batch no. 290121-B		

1	fHbp V2.19 M6 PorA$^{316\text{-}320\ exp\ 1.4}$	100 µg/mL
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Sodium Chloride	0.9%v/v
4	Phosphate buffer	2.5mM
5	Water for Injection	q.s
Result	**pH**	**7.53**
	Zeta potential	**-9.76 mV**
Batch no. 290121-C		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V2.19 M6 PorA$^{316\text{-}320\ exp\ 1.4}$	100 µg/mL
2	Adjuphos (Aluminium Phosphate)	2.27 mg/mL
3	Sodium Chloride	0.9%v/v
4	Phosphate buffer	2.5mM
5	Water for Injection	q.s
Result	**pH**	**6.94**
	Zeta potential	**-17.3 mV**

Conclusion: Three adjuvants were evaluated, namely, dmLT, Alhydrogel and Adjuphos. It is seen from Table-30 that Alhydrogel and Adjuphos adsorbed formulations gave satisfactory zeta potential than dmLT adsorbed formulation.

Alhydrogel has an isoelectric point (pI) at about 11.4 while Adjuphos has pI value in between 4.5-6.0. All the fHbp proteins have pI in the range of 5.5 to 6.5. Thus, to achieve optimum adsorption of all proteins on adjuvant in the vaccine formulations, Alhydrogel was used for further studies.

ii) Buffer and Sugar optimization:

Table-31: Buffer and sugar optimization		
Batch no. 051021-A		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA$^{307\text{-}311\ Exp1.9}$	100 µg/mL

2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Sodium Chloride	0.9%v/v
4	Sucrose	4%v/v
5	Water for Injection	q.s
Result	**pH**	**7.08**
	Zeta potential	**9.55 mV**
Batch no. 051021-B		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA$^{307\text{-}311\ Exp1.9}$	100 µg/mL
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Sodium Chloride	0.9%v/v
4	Mannitol	4%v/v
5	Water for Injection	q.s
Result	**pH**	**7.0**
	Zeta potential	**10.7 mV**
Batch No. 061021		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA$^{307\text{-}311\ Exp1.9}$	100 µg/mL
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Sodium Chloride	0.9%v/v
4	Mannitol	4%v/v
5	Phosphate buffer, pH7.4	5mM
6	Water for Injection	q.s
Result	**pH**	**8.0**
	Zeta potential	**-30.4 mV**

It is seen from Table-31 that the desired Zeta potential was not obtained for Batch No. 051021-A and Batch No. 051021-B, when sodium chloride and sucrose was used in the formulation. Sucrose was used during the initial stages of formulating the composition.

However, compositions with sucrose did not result in optimum physicochemical characters and hence was not included in the subsequent compositions.

When phosphate buffer was added to the formulation (Batch No. 061021), an improvement in the Zeta potential was observed, however, the pH value was not in the expected range. Further studies were conducted using mannitol and phosphate buffer. The details of the formulation and the result obtained is summarized in Table-32.

Table-32: Buffer and sugar optimization using mannitol and phosphate buffer		
Batch No.071021		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA$^{307\text{-}311\ Exp1.9}$	100 µg/mL
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Mannitol	5%v/v
4	Phosphate buffer, pH7.2	5mM
5	Water for Injection	q.s
Result	**pH**	**7.48**
	Zeta potential	**-18.8 mV**
	Osmolality	**300 mOsmol/kg**

It is seen from Table-32 that the pH, Osmolality and Zeta potential was in the expected range, when mannitol and phosphate buffer was used in the formulation. It is also observed that increasing mannitol concentration from 4% to 5% resulted in optimum osmolality of the formulation.

iii) Individual chimeric protein formulations:

Similar studies were carried out for the remaining proteins. The formulation detail and the result obtained are summarized in Table-33.

Table-33: Formulation detail for individual proteins										
Protein	**Formulation**						**result**			
	Protein (µg/ mL)	Alhydrogel (Aluminium hydroxide) (mg/ mL)	Mannitol (% v/v)	Phosphate buffer, pH7.2 (mM)	Polysorbate-20 (mg/ mL)	WFI	pH	Zeta potential (mV)	Osmolality (mOsmol/kg)	

fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6) (Batch No. 111021)	100	2.89	5	5	0.05	q.s	7.33	-23.2	357
fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7) (Batch No. 121021-A)	100	2.89	5	5	0.05	q.s	7.71	- 24.0	388
fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10) (Batch No. 121021-B)	100	2.89	5	5	0.05	q.s	7.56	-25.2	398
fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8) (Batch No. 141021)	100	2.89	5	5	0.05	q.s	7.56	-24.4	370

It is seen that using the formulation comprising mannitol (5% v/v), polysorbate 20 (0.05 mg/ mL) as provided in Table-33 resulted in optimum physicochemical parameters for all the individual proteins.

iv) Formulation comprising multiple chimeric proteins:

Similar to the individual protein formulation, physicochemical properties of formulation comprising the combination of proteins were determined. The formulation detail and result obtained are summarized in Table-34. The proteins were individually adsorbed on the adjuvant and then added to the excipient mixture to obtain the formulation.

Table-34: Vaccine formulation comprising optimized excipients

Batch No. 161021

Sr. No.	Formulation	Concentration
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	25µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	25µg/mL
3	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	25µg/mL
4	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	25µg/mL
5	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
6	Mannitol	5%v/v
7	Phosphate buffer, pH7.2	5mM
8	Polysorbate 20	0.05 mg/ mL

9	Water for Injection	q.s	
		Initial	**Day 7 (2-8°C)**
Result	pH	7.52	7.52
	Zeta potential	-27.0 mV	-26.1 mV
	% Adsorption	92.8%	82.5%

It is seen from Table-34 that the formulation was stable when stored at 2-8°C for about 7 days. Further, pH, Zeta potential, and % adsorption remained stable. Hence this combination of excipients in the formulation was taken for further scaling up.

v) Optimization of chimeric protein concentration:

Studies were conducted using higher concentration of the chimeric protein to determine the effect on pH, osmolality, and Zeta potential. The formulation detail and result obtained are summarized in Tables 35a and 35b.

Table-35a: Study on % adsorption		
Batch No. 251021		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA 307-311 exp 1.9fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	400 µg/mL
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Mannitol	5%v/v
4	Phosphate buffer, pH7.2	5mM
5	Polysorbate 20	0.05 mg/ mL
6	Water for Injection	q.s
Result	pH	7.26
	Zeta potential	-15.3 mV
	% Adsorption	93.3%
	% Adsorption- day 7	76.2%

Table-35b: Study on Zeta potential		
Batch No. 261021		
Sr. No.	**Formulation**	**Concentration**
1	fHbp V1.14 PorA 307-311 exp	400 µg/mL

	1.9fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	
2	Alhydrogel (Aluminium hydroxide)	2.89 mg/mL
3	Mannitol	5%v/v
4	Phosphate buffer, pH7.2	5mM
5	Polysorbate 20	0.05 mg/ mL
6	Water for Injection	q.s
Result	pH	7.45
	pH – Day3	7.52
	Zeta potential	-17.2 mV
	Zeta potential –Day3	-21.6 mV

It is seen from Tables 35a and 35b that the formulations at higher protein in combination with Phosphate buffer, polysorbate 20 and 5% Mannitol imparted optimum pH, osmolality, and Zeta potential.

vi) Optimization of concentration of excipients:

Studies were carried out to optimize the concentration of excipients. The formulation detail and the result obtained are summarized in Tables 36 to 39.

Table-36: Vaccine formulation comprising higher amount of protein along with excipients

Batch No. 180123

Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	4%v/v
7	Phosphate buffer, pH7.2	10mM
8	Polysorbate 20	0.05 mg/mL

9	2 phenoxyethanol	0.5% w/v
10	Water for Injection	q.s
Result		Initial
	pH	7.51
	Zeta potential (mV)	-23.8
	Osmolality (mOsmol/kg)	285
	% Adsorption	79

Table-37: Vaccine formulation with polysorbate 20		
Batch No. 230123 - Formulation 1		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	4.5%v/v
7	Phosphate buffer, pH7.0	5mM
9	Polysorbate 20	0.05 mg/mL
10	2phenoxyethanol	0.5% w/v
11	Water for Injection	q.s
Initial Result	pH	7.47
	Zeta potential (mV)	-22.5
	Osmolality (mOsmol/kg)	283
	% protein adsorption	92.2

Table-38: Vaccine formulation without polysorbate 20		
Batch No. 230123 – Formulation 2		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO.	120 µg/mL

	1, 6)	
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	4.5% v/v
7	Phosphate buffer, pH7.0	5mM
8	2 phenoxyethanol	0.5% w/v
9	Water for Injection	q.s
Initial Result	pH	7.48
	Zeta potential (mV)	-25.5
	Osmolality (mOsmol/kg)	290
	% protein adsorption	94.5

Table-39: Stability data for above Formulation 1 and Formulation 2

Sr. No.	Batch No.	Test Parameters	Initial	Accelerated condition (25°C)			Long term condition (2-8°C)		
				1M	3M	6M	1M	3M	6M
1	230123 – Formulation 1 (with PS20)	% Protein adsorption	92.2	87.8	84.2	79.8	91.6	87.1	82.7
		pH	7.47			7.90			7.79
		Zeta Potential (mV)	-22.5			-26.8			-25.5
		Osmolality (mOsmol/kg)	283			-			-
		PSD (Zavg, nm)	4919			1737			1987
2	230123 – Formulation 2 (without PS20)	% Protein adsorption	94.5	89.4	86.3	82.0	94.2	89.0	85.0
		pH	7.48			7.95			7.88
		Zeta Potential (mV)	-25.5			-28.9			-25.0
		Osmolality (mOsmol/kg)	290			-			-
		PSD (Zavg, nm)	3195			1914			2115

PSD= particle size distribution

It is seen from Tables 36 to 39 that using the combination of 5% v/v mannitol; 0.05 mg/ mL polysorbate 20; 5 mM phosphate buffer (pH 7.0) and 0.5 % w/v 2-phenoxyethanol in combination with 120 µg/ mL of each chimeric protein results in optimum physicochemical characteristics.

vii) Optimization of pH:

Studies were carried out to check the impact of pH on adsorption. The formulation detail and result obtained are summarized in Tables 40 and 41.

Table-40: Impact of pH on adsorption (pH of phosphate buffer: > 7)		
Batch No. 150323 – Formulation 1		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	5%v/v
7	Phosphate buffer, pH7.4	5mM
8	Polysorbate 20	0.05 mg/mL
9	2-phenoxyethanol	0.5% w/v
10	Water for Injection	q.s
Initial Result	pH	7.83
	Zeta potential (mV)	-21.7
	PSD (Zavg.nm)	1902
	% protein adsorption	85.7

Table-41: Impact of pH on adsorption (pH of phosphate buffer: > 7)		
Batch No. 150323 – Formulation 2		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5,	120 µg/mL

	10)	
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	5%v/v
7	Phosphate buffer, pH7.4	5mM
8	Polysorbate 20	0.05 mg/mL
9	2-phenoxyethanol	0.5% w/v
10	Water for Injection	q.s
Initial Result	pH	7.86
	Zeta potential (mV)	-21.3
	PSD (Zavg.nm)	2627
	% protein adsorption	86.9

It is seen from Tables 40 to 41 that increase in the pH of phosphate buffer to 7.4 resulted in an increased pH of the final formulation (7.83 to 7.86). Further, increase in the pH of the phosphate buffer resulted in decreased protein adsorption from 94 % to 85%.

Table-42: Impact of pH on adsorption (pH of phosphate buffer: 7)		
Batch No. 250423 – Formulation 1		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	5%v/v
7	Phosphate buffer, pH7.0	5mM
8	Polysorbate 20	0.05 mg/mL
9	2-phenoxyethanol	0.5% w/v
10	Water for Injection	q.s
Result		Initial
	pH	7.53
	Zeta potential (mV)	-19.1

	PSD (Zavg, nm)	3883
	% protein adsorption	88
	Osmolality (mOsmol/kg)	385

Table-43: Impact of pH on adsorption (pH of phosphate buffer: 7)		
Batch No. 250423 – Formulation 2		
Sr. No.	**Formulation**	**Concentration**
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL
4	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	120 µg/mL
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL
6	Mannitol	5%v/v
7	Phosphate buffer, pH7.0	5mM
8	Polysorbate 20	0.05 mg/mL
9	2-phenoxyethanol	0.5% w/v
10	Water for Injection	q.s
Result		Initial
	pH	7.46
	Zeta potential (mV)	-18.8
	PSD (Zavg, nm)	2136
	% protein adsorption	92.3
	Osmolality (mOsmol/kg)	373

It is seen from Tables 42 to 43 that when phosphate buffer of pH 7.0 was used for the formulation, a final pH in the range of 7.3 to 7.5 was achieved along with an increase in protein adsorption.

Example-4: Stability studies of the vaccine formulations comprising recombinant protein/ modified fHbp fusion protein

Stability studies at 2-8°C for 2 batches for 6 months and 10 months were carried out. The formulation detail and the result obtained are summarized in Table-44.

Table-44: Stability studies at 2-8°C

Sr. No.	Formulation	Concentration			
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL			
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL			
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL			
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 4, 9)	120 µg/mL			
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL			
6	Mannitol	50 mg/mL			
7	Phosphate buffer, pH 7.0	5mM			
8	Polysorbate 20	0.05 mg/mL			
9	2-phenoxyethanol	5 mg/mL			
10	Water for Injection	q.s			
Result	**Batch No.'s ⟹**	**090823 – Formulation 1**		**250423 – Formulation 2**	
	Test Parameters ⇩	**Initial**	**6M (2-8°C)**	**Initial**	**10M (2-8°C)**
	% protein adsorption	84.8	90.6	88	88.6
	pH	7.65	7.68	7.53	7.80
	Zeta potential (mV)	-20.6	-24.2	-19.1	-24.1
	PSD (Zavg, nm)	3228	1679	3883	1761
	Osmolality (mOsmol/kg)	-	330	-	385

Conclusion: It is seen from Table-44 that the % protein adsorption, pH, Zeta potential and PSD of the vaccine formulations comprising the recombinant proteins/ modified fHbp fusion proteins are within the desired range for up to 6 months at 2-8°C.

Example-5: Immunogenicity studies of formulations comprising recombinant/ chimeric proteins

Details of biological material used are provided below:

N. meningitidis serogroup B strains:

Glycerol stocks of *N. Meningitidis* were received from professor Chris Tang (Sir William Dunn School of Pathology, UK). Four strains of N. meningitidis serogroup B were received. The strain details are provided below and further summarized in Table-45.

1. N. meningitidis M08.240157 (fHbp V1.1:PorA VR2 16)
2. N. meningitidis M17. 240832 (fHbp 1.4: PorA VR2 4)
3. N. meningitidis M17. 240156 (fHbp 3.45: PorA VR2 14)
4. N. meningitidis M18 240043 (fHbp 1.15: PorA VR2 15-11)

Table-45: N. meningitidis serogroup B strains used for immunogenicity studies

Sr. No	PubMLST ID	Isolate	fHbp peptide	NadA peptide	NHBA peptide	PorA_VR1	PorA_VR2	ST (MLST)	Clonal complex (MLST)
1.	40818	M08240157	1	1	3	7	16	32	ST-32 complex
2.	60604	M17240832	4	0	2	7-2	4	1194	ST-41/44 complex
3.	53319	M17240156	45	0	18	22	14	213	ST-213 complex
4.	60955	M18240043	15	0	21	19-1	15-11	269	ST-269 complex

Details of the formulations used for immunogenicity studies are summarized in Table-46.

Table-46: Vaccine formulations for immunogenicity studies

Sr. No.	Formulation	Formulation 1 Batch No. 250423	Formulation 2 Batch No. 250423	Placebo
1	fHbpV3.45 M5:PorA316-320/exP1.14 (SEQ ID NO. 1, 6)	120 µg/mL	120 µg/mL	NA
2	fHbpV2.19 M6:PorA316-320/exP1.4 (SEQ ID NO. 2, 7)	120 µg/mL	120 µg/mL	
3	fHbpV1.14:PorA307-311/exP1.9 (SEQ ID NO. 3, 8)	120 µg/mL	120 µg/mL	
4	fHbpV1.1:PorA307-311/exP1.4 (SEQ	120 µg/mL	-	

	ID NO. 4, 9)			
	fHbpV1.1:PorA307-311/exP1.9 (SEQ ID NO. 5, 10)	-	120 µg/mL	
5	Alhydrogel (Aluminium hydroxide)	3.4 mg/mL	3.4 mg/mL	3.4 mg/mL
6	Mannitol	5%v/v	5%v/v	5%v/v
7	Phosphate buffer, pH7.0	5mM	5mM	5mM
8	Polysorbate 20	0.05 mg/mL	0.05 mg/mL	0.05 mg/mL
9	2-phenoxyethanol	0.5% w/v	0.5% w/v	0.5% w/v
10	Water for Injection	q.s	q.s	q.s
Result		Initial	Initial	Initial
	pH	7.53	7.46	7.16
	Zeta potential (mV)	-19.1	-18.8	-19.5
	PSD (Zavg, nm)	3883	2136	3188
	% protein adsorption	88	92.3	NA

Serum Bactericidal Assay

Procedure for hSBA against Men B Strains

Preparation by working assay stocks for SBA

The master stock vial was retrieved from the deep freezer, culture was quickly streaked onto an already-dried blood agar plate (Columbia blood agar with 5% Horse blood). Plate was incubated at 37°C with 5 % CO_2 overnight. Approximately 50 colonies were picked with a sterile loop and re-suspended (thus creating a heavy suspension) in a sterile 50 ml centrifuge tube containing 15 ml of BHI broth with glycerol. Suspension was aliquoted as 0.25 ml volumes into 1.8 ml cryo vials. Labelled vials were stored in -70°C deep freezer.

Assay procedure for Serum Bactericidal Assay for titration of antibodies against Men B strains:

N. meningitidis B strains (Working stock) were streaked on to a single blood agar plate and incubated overnight at 37°C with 5% CO_2. Culture is again streaked onto a fresh blood agar plate for confluent growth, as a square at the center of the plate. Plate was Incubated in a CO_2 incubator at 37°C with 5% CO_2 for 4 hours (+ 15 minutes) for all Men B strains. Growth swept from the center of the plate was suspended into 5 ml of Hanks Balanced salt solution/buffer, Invitrogen, with Ca+2 and Mg+2 to make a suspension. 1 ml of the suspension was used to read absorbance at 650nm. Suspension was adjusted by dilution to, 650nm= 0.1 OD, followed by a 1/10 and then a 1/250 dilution with the Buffer. 20 µl of

Buffer was added to all wells until column 11 of a 96 well microtiter plate. 20 µl of (Diluted or undiluted) test serum samples were added to 1st column of the plate; last two rows were kept reserved for quality control serum. 20 µl was serially diluted from 1st column to 2nd column and so on till 9th column, 20 µl from the 9th column is discarded. Columns, 10 and 11 were reserved for complement control. 10 µl of bacterial suspension already prepared was added to all wells until the 11th column. 10 µl of Human complement was added to all wells up to 10th column. 10 µl of heat inactivated (56°C for 30 mins) Human complement was added to all wells of the 11th column. Contents of the wells were mixed well, and the plate was kept for incubation at 37°C for 1 hour. After incubation, 10 µl contents of each well were spotted onto properly labelled blood agar plates. Blood agar plates were incubated in a CO_2 incubator at 37°C with 5% CO_2. Bacterial colonies were counted on the next day using an automated colony counter (Synbiosis – ProtoCOL3).

Study Details:

Immunogenicity study was conducted in Rabbits (New Zealand White) with 3 formulations/groups, G1-Trumenba (Pfizer), G2-Formulation 1, G3-Formulation 2, each formulation was injected into 4 male and 4 female rabbits. Injections were given on Day 0, 14 and 28 and bleeding was performed on D0 and D37. Sera samples were analysed for immunogenicity by hSBA (Serum bactericidal Assay using Human Complement) Full human dose were given to all rabbits. **Drug Product:** Meningococcal B vaccine (MenB) (250423-Formulation 1 and 2)

Study Design: The study design is summarized in Table-47.

Table-47: Treatment Groups in Immunogenicity Study

Sr. No.	Group ID	Test Compound	Dose (mcg) / inj.	No.s	Inj. Route	Inj. Vol. (µL)	Day of Dosing	Sera Collection Day	
			MenB					Day 0	Day 37
1	G1	Trumenba	120	8	IM	500	1, 14, 28	8	8
2	G2	Formulation 1	240	8	IM	500	1, 14, 28	8	8
3	G3	Formulation 2	240	8	IM	500	1, 14, 28	8	8
Total Rabbits				**24**	**-**			**24**	**24**
Total Sera samples								**48**	

Criteria for significant difference among groups:

2-fold change in titers is considered as assay variation and a $\geq$ 4-fold difference in titers amongst comparing groups is considered as significant.

Purpose and Scope:

Purpose of the study was to compare immunogenic response for individual Men B formulations – Formulation 1 and Formulation 2 along with Trumenba against MenB strains - M 08240157(fhbp 1.1), M 17240832 (fhbp 1.4), M 17240156 (fhbp 3.45) and M 18240043 (fhbp 1.15) hSBA. The results obtained are illustrated in Table-48.

Table-48: hSBA results for formulations against different Men B strains

Formulation	Men B strains											
	M08240157			M17240832			M17240156			M18240043		
	D0	D37	F.D.	D0	D37	F.D.	D0	D37	F.D.	D0	D37	F.D.
Trumenba-G1	9	32	4	9	117	13	235	1878	8	9	2233	248
Formulation 1-G2	9	128	14	6	41	7	152	1024	7	6	215	36
Formulation 2-G3	10	64	6	4	29	7	152	790	5	4	279	70
F.D = Fold Difference for titers upon comparing with baseline SBA(D37/D0) titers obtained. All values given are GMT for each of the data set												

The graphical representation with individual data points and GMT highlighted for each day of bleeding is provided in Figure-26.

It is seen from the hSBA data that the formulations (Formulation 1/ Quad 1 and Formulation 2/ Quad 2) comprising the recombinant proteins/ modified fHbp fusion proteins of the present disclosure are highly immunogenic and capable of eliciting protective SBA titer against different (M 08240157, M 17240832, M 17240156, and M 18240043) strains of N. meningitides.

i) ELISA for Assessing the binding of human complement factor-H to fHbp-PorA chimeric proteins as compared to the recombinant wild-type of Men B samples

PURPOSE:

The purpose of this study is to describe the method for assessing the binding of human complement factor-H to fHbp-PorA chimeric proteins as compared to the recombinant wild-type of Men B samples in Monoclonal Antibodies IPQC Laboratory (MAb IPQC).

PROCEDURE:

Chemicals and Reagents required are provided in Table-49.

Table-49: List of Chemicals and reagents

Sr. No.	Chemical Name	Vendor/ Brand	Catalog No.

1.	Complement Factor H from human plasma	Sigma Aldrich	C5813
2.	OX24 anti-factor H monoclonal antibody	Abcam	ab118820
3.	Polyclonal goat anti-mouse HRP secondary antibody	Sigma Aldrich	A4416
4.	Tween20	Sigma Aldrich	P7949
5.	Bovine Serum Albumin (BSA)	Sigma Aldrich	A7030
6.	Development reagent	R&D systems	DY999
7.	Stop solution	R&D systems	DY994
8.	PBS buffer tablet (pH 7.4 ±0.05)	Gibco	18912-014

For all the above chemicals, equivalent make from other brands can also be used.

Equipment required:

- ELISA plate reader capable of measuring wavelengths at 450 nm and 630 nm.
- Microplate washer
- Incubator capable of achieving and maintaining 37°C
- Refrigerator

Consumables required:

- NUNC-Immunoplate, Make: Thermo Scientific; Cat. No.: 442404 or equivalent.
- Plate sealing tape
- Reagent reservoirs 50 mL
- Glassware as applicable
- Micropipettes (single channel and multi-channel) and respective tips
- Vortex mixture
- Microcentrifuge tubes - 15 mL and 50 mL

Preparation of Reagent and solutions:

- **Coating Buffer: 1X PBS pH 7.4**

  Dissolve 2 PBS tablets in 800 µL of WFI, and then make up the volume to 1 L. Store at room temperature (RT), use before 1 month of date of preparation.

- **Wash Buffer: 1X PBST (0.05% Tween20 in 1X PBS pH 7.4)**

  Prepare 1 L of PBS as mentioned above. Add 0.5 mL of Tween 20 solution to 999.5 mL of 1X PBS and mix using a magnetic stirrer. Store at room temperature (RT), use before 1 month of date of preparation. This 1X

PBST is also used as diluent for blocking solution and antibody preparation.

- **Blocking: 4% BSA in PBST**

  Accurately weigh 4.0 g of BSA and dissolve it in 100 mL of 1X PBST.

  TMB solution: 2 component mixture

  Accurate measure equal volumes of solution A and B (provided as development reagent). Equilibrate to RT (store in dark). Prepare freshly, mix gently before use.

  Note: The procedure given above for preparation of reagents and solutions is for illustration purpose only. The actual volume/amount of chemical/reagent should be calculated according to the number of assay plates.

Method:

- **Coating of Antigen:**
  - Label the side of the 96 well plates with the Antigen details, analyst initials and date when the plate is coated.
  - Determine the volume of the respective antigen and coating buffer required to coat the desired number of plates.
  - Coat each well of an ELISA plate with 5.0 µg of antigen (fHbp-PorA chimera), diluted in phosphate buffered saline (1X PBS) to a final concentration of 0.1 µg/µL and with the help of a multi-channel pipette, add 50 µL of the diluted antigen to each well of 96-well Nunc flat bottom plates.
  - As a control, add 50.0 µL PBS alone to wells.
  - Incubate the sealed plate at 2-8°C overnight.
- **Washing:**
  - Remove coated 96 well microtiter plate, place the plate in microplate washer and perform the wash procedure by selecting the pre-feeded programme for 3 washes.
  - For manual washing, empty all wells by discarding the contents into a sink, add 300 µL/well of wash buffer/1X PBST, allow it to soak for few seconds and empty all wells into a sink. Tap gently on link free tissue

paper to make sure all the wells are completely empty. Repeat the wash process twice.

- **Blocking:**
  - The following day remove unbound antigen by washing the plate three times with 300 µL/well of 1X PBST.
  - Block wells with 200 µL 4% BSA in PBST. Incubate the plate at 37°C for 1 hour.
- **Addition of Human complement factor-H:**
  - Remove unbound BSA by washing the plate three times with 300 µL/well of 1X PBST.
  - Human complement factor-H dilution scheme is summarized in Table-50:

Table-50: Human complement factor-H dilution scheme

Dilution number	Volume from stock (µL)	Volume of diluent (µL)	Final Volume (µL)	Resulting concentration (µg/mL)
1	30.0 µL from 1.0 mg/mL	1470.0 µL	1500.0 µL	20.0 µg/mL
2	250.0 µL from 20.0 µg/mL	250.0 µL	500.0 µL	10.0 µg/mL
3	200.0 µL from 20.0 µg/mL	300.0 µL	500.0 µL	8.0 µg/mL
4	150.0 µL from 20.0 µg/mL	350.0 µL	500.0 µL	6.0 µg/mL
5	100.0 µL from 20.0 µg/mL	400.0 µL	500.0 µL	4.0 µg/mL
6	50.0 µL from 20.0 µg/mL	450.0 µL	500.0 µL	2.0 µg/mL
7	25.0 µL from 20.0 µg/mL	475.0 µL	500.0 µL	1.0 µg/mL
8	50.0 µL from 2.0 µg/mL	450.0 µL	500.0 µL	0.2 µg/mL
9	50.0 µL from 1.0 µg/mL	450.0 µL	500.0 µL	0.1 µg/mL
10	50.0 µL from 0.2 µg/mL	450.0 µL	500.0 µL	0.02 µg/mL

  - Add 50.0 µL of factor H diluted to appropriate final concentrations (follow the dilution scheme as shown in the Table-50, the following are the final concentrations of factor H, 0.0 µg, 0.001 µg, 0.005 µg, 0.01 µg, 0.05 µg, 0.1 µg, 0.2 µg, 0.3 µg, 0.4 µg, 0.5 µg and 1.0 µg of protein in 50.0 µL) and incubate the plate at 37°C for 1 hour.
- **Addition of Primary Antibody:**
  - Remove unbound factor H by washing the plate three times with 300 µL/well of 1X PBST.

- Add 50.0 µL of OX24 mAb diluted to 1/10,000 in PBST and incubate the plate at 37°C for 1 hour.

- **Addition of Secondary Antibody:**
  - Remove unbound primary antibody (OX24 mAb) by washing the plate three times with 300 µL/well of 1X PBST.
  - Add 50.0 µL of secondary antibody diluted to a final concentration of 1/10,000 in PBST and incubate the plate at 37°C for 1 hour.

- **Addition of TMB substrate:**
  - Remove unbound secondary antibody by washing the plate three times with 300 µL/well of 1X PBST.
  - Develop the plate by adding 100.0 µL of development reagent (development reagent in a 1:1 mix of solution A and solution B). Incubate the plate at 25°C/RT for 20 min and stop by adding 50.0 µL stop solution, which turns reactions yellow.
  - Read the absorbance of the developed wells on a plate reader at Absorbance at 450nm & 630nm. Normalized Absorbance (A450nm - A630nm) is used to plot the graphs using excel and Graph-pad PRISM.

Figure-27 and Table-51 illustrate the representative data for binding of human complement factor-H to wildtype fHbp as compared to Men B chimeric proteins.

Table-51: Binding of human complement factor-H

Chimeric Protein	Human Complement factor-H Concentration	SIIPL Chimera	Oxford Chimera	Oxford WT	Fold Potency compared to Oxford ChA	Fold Potency compared to Oxford Wt	Difference (compared to Oxford Wt)
fHbp V3.45 M5: PorA 316-320/exP1.14	4 µg/mL	0.171	NA	1.244	NA	13.75	86.25
fHbp V2.19 M6: PorA 316-320/exP1.4	4 µg/mL	0.064	0.080	1.176	80.00	5.44	94.56
fHbp V1.14 : PorA 307-311/exP1.9	4 µg/mL	0.095	0.074	1.036	129.25	9.17	90.83
fHbp V1.1 : PorA 307-311/exP1.4	4 µg/mL	0.065	0.055	0.982	117.27	6.57	93.43

fHbp V1.1 : PorA $^{307-311/exP1.9}$	4 µg/mL	0.061	0.060	0.982	101.67	6.21	93.79

Conclusion: It is seen from Table-51 and Figure-27 that the recombinant protein/ modified fHbp fusion protein of the present invention exhibits more than 80% reduction in human complement factor H binding when compared to wild type fHbp protein.

Example-6: Combination formulations comprising recombinant protein/ modified fHbp fusion protein formulation

Physicochemical characterization of combination formulation:

The vaccine formulation of the present invention comprising recombinant proteins/ modified fHbp fusion proteins from N. meningitidis serogroup B was found to be stable and immunogenic. Further, combination formulations comprising the vaccine formulation along with other N. meningitidis serogroups were studied to find out the impact on physicochemical characterization, stability, and immunogenicity.

Applicant's MenFive formulation comprising polysaccharide-protein conjugates against N. meningitidis serogroups ACYWX was used for the study. The lyophilized MenFive vaccine was reconstituted with the MenB formulation and stored at room temperature for 2 hours to study the impact on physicochemical parameters of overall reconstituted solution. The study was done to evaluate stability of reconstituted solution during animal injections and mimic routine clinical practice.

MenB formulation from Batch No. 090823 and MenFive formulation (Batch No. 2352M001: 5-dose lyophilized vial was used in this study, contents per vial: Each antigen (A, C, Y, W, X) = 32µg; Sucrose = 15mg; Trisodium citrate = 2.5 mg; Tris buffer = 0.61 mg) was used for the study. The test parameters and result obtained are summarized in Table-52.

Table-52: Physicochemical characterization of combination formulations

Sr. No.	Admixture	Test Parameters	Initial	2 hrs at RT
1	MenFive (B#2352M001) + Placebo for MenB (B#090823)	pH	7.30	7.30
		Zeta Potential (mV)	-33.3	-32.8
		PSD (Zavg, nm)	1550	1938
2	MenFive (B#2352M001) + MenB (B#090823-Formulation 1)	pH	7.64	7.63
		Zeta Potential (mV)	-41.3	-41.6

		PSD (Zavg, nm)	1912	2302
3	MenFive (B#2352M001) + MenB (B#090823-Formulation 2)	pH	7.65	7.66
		Zeta Potential (mV)	-35.6	-35.8
		PSD (Zavg, nm)	2421	2447

Conclusion**:** It is seen from Table-52 that the reconstituted solution was stable physico-chemically for 2 hours and also visually no particle aggregation / settling was seen.

Hence, further studies were planned for determining immunogenicity and non-interference of the MenFive + MenB combination formulations.

Immunogenicity and non-interference studies of combination formulation:

Details of biological material used are provided below:

N. meningitidis serogroup B strains:

The N. meningitidis serogroup B strains used were same as those used in Example-5.

N. meningitidis serogroup A, C, W, Y and X) strains:

Glycerol stocks of *N. Meningitidis* were received from Professor Ray Borrow (Health Protection Agency, Manchester Laboratory UK) in July 2012. Five serogroups of N. *Meningitidis* were received as follows:

1. N. meningitidis serogroup A, F8238
2. N. meningitidis serogroup C11, M05 240852
3. N. meningitidis serogroup W135, M01 0240070
4. N. meningitidis serogroup X, BF2/97
5. N. meningitidis serogroup Y, M03 0241125

Procedure for hSBA against Men B Strains:

Preparation by working assay stocks for SBA

The master stock vial was retrieved from the deep freezer, culture was quickly streaked onto an already-dried blood agar plate (Columbia blood agar with 5% Horse blood). Plate was incubated at 37°C with 5 % CO_2 overnight. Approximately 50 colonies were picked with a sterile loop and re-suspended (thus creating a heavy suspension) in a sterile 50 ml centrifuge

tube containing 15 ml of BHI broth with glycerol. Suspension was aliquoted as 0.25 ml volumes into 1.8 ml cryo vials. Labelled vials were stored in -70°C deep freezer.

Assay procedure for Serum Bactericidal Assay for titration of antibodies against Men B strains:

N. meningitidis B strains (Working stock) were streaked on to a single blood agar plate and incubated overnight at 37°C with 5% CO_2. Culture is again streaked onto a fresh blood agar plate for confluent growth, as a square at the center of the plate. Plate was Incubated in a CO_2 incubator at 37°C with 5% CO_2 for 4 hours (+ 15 minutes) for all Men B strains. Growth swept from the center of the plate was suspended into 5 ml of Hanks Balanced salt solution/buffer, Invitrogen, with Ca+2 and Mg+2 to make a suspension. 1 ml of the suspension was used to read absorbance at 650nm. Suspension was adjusted by dilution to, 650nm= 0.1 OD, followed by a 1/10 and then a 1/250 dilution with the Buffer. 20 µl of Buffer was added to all wells until column 11 of a 96 well microtiter plate. 20 µl of (Diluted or undiluted) test serum samples were added to 1st column of the plate; last two rows were kept reserved for quality control serum. 20 µl was serially diluted from 1st column to 2^{nd} column and so on till 9^{th} column, 20 µl from the 9^{th} column is discarded. Columns 10 and 11 were reserved for complement control. 10 µl of bacterial suspension already prepared was added to all wells until 11^{th} column. 10 µl of Human complement was added to all wells up to 10^{th} column. 10 µl of heat inactivated (56°C for 30 mins) Human complement was added to all wells of the 11^{th} column. Contents of the wells were mixed well, and the plate was kept for incubation at 37°C for 1 hour. After incubation, 10 µl contents of each well were spotted onto properly labelled blood agar plates. Blood agar plates were incubated in a CO_2 incubator at 37°C with 5% CO_2. Bacterial colonies were counted on next day using an automated colony counter (Synbiosis – ProtoCOL3).

Procedure for hSBA against MenFive serogroups (A, C, W, Y and X):

Preparation by working assay stocks for SBA

The master stock vial was retrieved from the deep freezer, culture was quickly streaked onto an already-dried blood agar plate (Columbia blood agar with 5% Horse blood). Plate was incubated at 37°C with 5 % CO_2 overnight. Approximately 50 colonies were picked with a sterile loop and re-suspended (thus creating a heavy suspension) in a sterile 50 ml centrifuge tube containing 15 ml of BHI broth with glycerol. Suspension was aliquoted as 0.25 ml volumes into 1.8 ml cryo vials. Labelled vials were stored in -70°C deep freezer.

Assay procedure for Serum Bactericidal Assay for titration of antibodies MenFive serogroups (A, C, W, Y and X):

N. meningitidis serogroups- A, C, W, Y and X, working stock were streaked on to a single blood agar plate and incubated overnight at 37°C with 5% CO_2. Culture is again streaked onto a fresh blood agar plate for confluent growth, as a square at the center of the plate. Plate was Incubated in a CO_2 incubator at 37°C with 5% CO_2 for 4 hours (±15 minutes) for serogroup C, W, Y, X and for Serogroup A, incubation of 3 hours (±15 minutes). Growth swept from the center of the plate was suspended into 5 ml of Hanks Balanced buffer with Ca^{+2} and Mg^{+2} (Hanks balanced salt solution, Invitrogen) for serogroup A, Y and X and Ca- and Mg- with BSA for serogroup C and W, to make a suspension. 1 ml of the suspension was used to read absorbance at 650nm. Suspension was adjusted by dilution to, 650nm= 0.1 OD, followed by a 1/10 and then a 1/250 dilution with the Buffer. 20 µl of Buffer was added to all wells until column 11 of a 96 well microtiter plate. 20 µl of (Diluted or undiluted) test serum samples are added to 1st column of the plate; last two rows were kept reserved for quality control serum. 20 µl was serially diluted from 1st column to 2nd column and so on till 9th column, 20 µl from the 9th column is discarded. Columns 10 and 11 are reserved for complement control. 10 µl of bacterial suspension already prepared was added to all wells until 11th column. 10 µl of Human complement was added to all wells up to 10th column. 10 µl of heat inactivated Human complement was added to all wells of the 11th column. Contents of the wells were mixed well, and the plate was kept for incubation at 37°C for 1 hour. After incubation, 10 µl contents of each well were spotted onto properly labelled blood agar plates. Blood agar plates were incubated in a CO_2 incubator at 37°C with 5% CO_2. Bacterial colonies were counted on next day using an automated colony counter (Synbiosis – ProtoCOL3).

Study Details:

Immunogenicity study was conducted in Rabbits (New Zealand White) with 5 formulations/groups (G1-Placebo, G2-MenFive, G3-MenFive+MenB-Formulation 1, G4-MenFive+MenB-Formulation 2 and G5-Menfive+Trumenba), each formulation was injected into 4 male and 4 female rabbits. Injections were given on Day 0, 14 and 28 and bleeding was performed on D0, D28 and D41, Sera samples were analyzed for immunogenicity by hSBA (Serum bactericidal Assay using Human Complement). Full human dose was given to all rabbits.

Drug Product: Meningococcal (A-TT, C-CRM, Y-CRM, W-CRM, X-TT) Polysaccharide

Conjugate Vaccine – 5 Dose vial (Men5) (2352M001) reconstituted with Meningococcal B vaccine (MenB) (090823-Formulation 1 and 2)

Study Design:

The study design is illustrated in Table-53.

Table-53: Treatment Groups in Immunogenicity Study

Sr. No.	Group ID	Test Compound	Dose (mcg) / inj.		No.	Inj. Route	Inj. Vol. (µL)	Day of Dosing	Sera Collection Day		
			Men5	MenB					Day 0	Day 28	Day 41
1	G1	Placebo for MenB formulation	-	-	8	IM	500	1, 14, 28	8	8	8
2	G2	Men5 + Saline	25	-	8	IM	500	1, 14, 28	8	8	8
3	G3	Men5 + MenB (Formulation 1)	25	240	8	IM	500	1, 14, 28	8	8	8
4	G4	Men5 + MenB (Formulation 2)	25	240	8	IM	500	1, 14, 28	8	8	8
5	G5	Men5 + Trumenba	25	120	5	IM	500	1, 14, 28	5	5	5
Total Rabbits					**37**	**-**			**37**	**37**	**37**
Total Sera samples									**111**		

Criteria for significant difference among groups:

2-fold change in titers is considered as assay variation and a > 4-fold difference in titers amongst comparing groups is considered as significant.

Purpose and Scope:

Purpose of the study was to compare immunogenic response for individual MenFive+MenB, admix formulations, against MenFive serogroups (A, C, W, Y and X) and MenB strains - M 08240157(fhbp 1.1), M 17240832 (fhbp 1.4), M 17240156 (fhbp 3.45) and M 18240043 (fhbp 1.15) on using hSBA. The scope also includes, to check for any interference/cross protection in response between the two individual vaccines formulations (MenFive and MenB).

The result obtained is summarized in Tables 54-55 and Figures 28-29.

Table-54: hSBA results for formulations against MenFive serogroups:

Formulation	MEN A				MEN C				MEN W				MEN Y				MEN X			
	D0	D28	D41	F.D.	D0	D28	D41	F.D.	D0	D28	D41	F.D.	D0	D28	D41	F.D.	D0	D28	D41	F.D.
Placebo	2	2	2	1.0	2	2	2	1.0	2	2	2	1.0	2	2	2	1.0	23	17	25	1.1
MenFive	3	939	609	181.1	3	197	140	41.6	3	512	181	53.8	3	304	256	76.1	29	558	152	5.2
MenFive+Formulation 1	2	1117	861	430.5	2	166	181	90.5	2	1024	470	235.0	2	256	166	83.0	45	558	431	9.5
MenFive+Formulation 2	2	1218	790	395.0	2	152	215	107.5	2	939	362	181.0	2	235	166	83.0	29	664	362	12.3
MenFive+Trumenba	2	388	194	97.0	2	223	512	256.0	2	891	388	194.0	2	446	1024	512.0	28	111	64	2.3

Table-55: hSBA results for formulations against MenB serogroups:

Formulation	M08240157				M17240832				M17240156				M18240043			
	D0	D28	D41	F.D.	D0	D28	D41	F.D.	D0	D28	D41	F.D.	D0	D28	D41	F.D.
Placebo	11	10	19	1.7	2	2	7	3.5	76	256	235	3.1	4	4	5	1.4
MenFive	15	10	38	2.6	5	2	13	2.5	99	304	304	3.1	7	5	4	0.5
MenFive+Formulation 1	16	27	108	6.8	6	21	41	6.6	117	558	609	5.2	6	45	197	31.9
MenFive+Formulation 2	16	10	64	4	5	23	35	6.7	140	790	861	6.2	7	64	117	17.4
MenFive+Trumenba	9	14	49	5.3	3	28	42	12.1	74	776	676	9.2	12	147	891	73.5

Conclusion: Following observations were made on the basis of the result obtained in Tables 54-55 and Figures 28-29.

Inference for MenFive serogroups: Titers obtained for all five serogroups (A, C, W, Y and X) for the combination formulations (MenFive+Formulation 1 and MenFive+Formulation 2) are comparable (within 2-fold) with the individual MenFive alone formulation, suggesting no loss in immunogenic response upon admixing with Men B component. The SBA titers have increased, number wise slightly, but no reduction is seen for any of the serogroups.

Inference for Men B Serogroups: Titers against Men B strains for MenFive+MenB formulations show a ≥ 4-fold rise in titers on comparing pre (D0) vs post (D41) titers, thus suggesting improved and acceptable immune response generation against 4 different strains of Men B.

Results suggest that the combination of MenFive with Men-B formulations does not result in any loss of immunogenic response, thus indicating no interference for the combination formulations for either of the vaccine components.

Example-7: Quantification of recombinant protein/ modified fHbp fusion protein using ELISA

Bicinchoninic Acid (BCA) method is routinely used for estimating total recombinant protein/ modified fHbp fusion protein content in the final vaccine formulation.

The vaccine formulation of the present invention comprises more than one recombinant protein/ modified fHbp fusion protein. BCA provides the total recombinant protein/ modified fHbp fusion protein quantification and is not able to provide the individual content of each protein present in the vaccine formulation.

As an alternative, ELISA was used to quantify the content of individual recombinant protein/ modified fHbp fusion protein in the vaccine formulation. This test may also be used as a batch release test.

The material used for ELISA for quantifying individual recombinant protein/ modified fHbp fusion protein is summarized in Table-56.

Table-56: Material used for protein quantification

Material Name	Make	Catalog Number
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates	Thermo Scientific	442404
BSA	Sigma	A7030
PBS	Gibco	18912-014
Tween-20	Sigma	P-7949
Anti-Factor H binding protein [JAR41], Rabbit IgG, Kappa	Absolute Antibody	Ab03894-23.0
Anti-Factor H binding protein from N. meningitidis [JAR5], Human IgG1,	Absolute Antibody	Ab00445-10.3

Kappa		
Anti-fHbp Antibody, clone JAR 11	Merck	MABF2668-100UG
Anti-Meningococcal Serosubtype P1.4 mAb	NIBSC	02/148
Anti-Meningococcal Serosubtype P1.14 mAb	NIBSC	03/142
Anti-Meningococcal Serosubtype P1.9 mAb	NIBSC	05/190
Polyclonal goat anti-mouse HRP secondary antibody	Sigma	A4416
TMB	Sigma	T0440-1L
HCl	Fisher scientific	29507

Assay procedure:

STEP-1 (Capture Antibody Coating): JAR-41 or JAR-5 antibody will be diluted using 0.1 % BSA in PBS and used for coating in range from 0.5 µg to 10 µg per well and load 100 µL per well on 96 well plate. The coating incubation will be from 2.5 hrs to 18 hrs at either room temperature or 2-8°C.

STEP-2 (Blocking): 300 to 320 µL of 1% BSA in PBS will be added to 96 well plate and incubated from 1 hr to 2.5 hrs at room temperature.

STEP-3 (Standard / Test Sample Addition): The reference standard chimeras will be serially diluted from 0.5mg/mL to 1ng/mL using 0.1 % BSA in PBS and 50 µL to 150 µL will be added to respective wells. The test samples will be diluted using 0.1 % BSA in PBS such that they will fall in the quantification range by loading from 50 µL to 150 µL. After addition of both the Standard and Test sample will be incubated from 0.5 hrs to 2.0 hrs at room temperature.

STEP-4 (Detection Antibody Addition): The detection antibody (Anti-POR-A P1.14 mAb / Anti-POR-A P1.4 mAb / Anti-POR-A P1.9 mAb / JAR-11 mAb) will be diluted anywhere between 100 fold and 100000 fold and load 100 µL per well on 96 well plate. The detection antibody incubation will be from 0.5 hrs to 2.0 hrs at room temperature.

STEP-5 (Secondary Detection Antibody Addition): The secondary detection antibody (goat anti-mouse HRP antibody) will be diluted anywhere between 100 fold and 100000 fold and

load 100 µL per well on 96 well plate. The secondary detection antibody incubation will be from 0.5 hrs to 2.0 hrs at room temperature.

STEP-6 (Substrate Addition & Stopping): The 100 µL of TMB substrate will be added to each well and incubated from 10 to 45 min at room temperature followed by stopping with addition of 100 µL of 1N HCl to each well. Immediately the plate will be read for absorbance at 450 /630.

Example-8: Estimation of protein in recombinant protein/ modified fHbp fusion protein using BCA

PURPOSE:

The purpose of this study is to provide the procedure for estimation of protein in Men B samples by 96 well plate Micro Bicinchoninic acid (BCA) method.

BCA is a method for quantifying the concentration of protein in a sample. Under alkaline conditions, Cu^{2+} reacts with proteins yielding Cu^{+}. This Cu^{+} generated reacts with bicinchoninic acid to form a purple coloured complex. The colour is developed after incubation at 37 °C. The coloured complex is detected using spectrophotometer at 562nm.

MATERIAL & EQUIPMENTS:

- Micro BCA Protein Assay Kit (Thermoscientific, 23235) containing
    a. Micro BCA Reagent A (MA)
    b. Micro BCA Reagent B (MB)
    c. Micro BCA Regent C (MC)
    d. Bovine Serum Albumin Standard ampules (2 mg/ml)
- Water for injection (WFI)
- Microfuge tubes (1.5 mL Eppendorf tubes)
- Pipettes and tips
- 96 well plate
- 96 well plate reader (TECAN, Infinite M200)
- Plate incubator (Biosan Thermoshaker)

TEST PROCEDURE:

<u>Preparation of standards and working reagent</u>**:**

- Set the incubator at 37°C.
- Prepare a stock of 200 µg/ml from the BSA standard (2mg/ml).

- Using the stock of 200 µg/ml prepare the standard curve as given in Table-57 and Figure-30. Prepare the standard curve each time the assay is to be performed.
- Prepare the working reagent by mixing 25 parts of Micro BCA reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1).

Table-57: Preparation of standard

Vial	Volume of Diluent (µL)	Volume (µL) and Source of BSA	Final BSA Concentration (µg/ml)
A	349.98	150.15 µl from 200 µg/ml stock	60
B	640	160 µl from 200 µg/ml stock	40
C	400	400 µl from vial B dilution	20
D	400	400 µl from vial C dilution	10
E	400	400 µl from vial D dilution	5
F	400	0	0

Sample Preparation:

The MEN-B formulated samples were analyzed as a) Sample without centrifugation, b) Supernatant after centrifugation, and c) Pellet after centrifugation.

a. **Sample without centrifugation:** The formulation samples are either taken as neat or diluted so as to fit in the standard curve.
b. **Supernatant:** 500 µl of each sample is taken and centrifuged at 8000 rpm for 5 minutes. After centrifugation, the supernatant is carefully transferred into a fresh tube. The collected supernatant is taken as a neat sample or diluted so as to fit in the curve.
c. **Pellet:** The pellet formed after centrifugation is re-suspended by adding 500 µl of WFI. These samples are diluted same as per **Sample as such (a).**

- Prepare the dilutions in duplicate for each sample and standards.
- Transfer 150 µl of standards and samples into the plate wells.
- Add 150 µl of the working reagent to each well and mix the plate thoroughly on a plate shaker for 30 seconds.
- Cover the plate and incubate at 37°C for 2 hours.
- Measure the absorbance at 562 nm and the result obtained is provided in Table-58.

Table-58: Absorbance at 562 nm

Std.	Conc (µg/mL)	Abs @ 562 nm_Read 1	Abs @ 562 nm_Read 2	Average Absorbance	Normalized Absorbance	Std Dev	CV %	Std Back calculated values	Std Recovery (%)

B	0.000	0.0994	0.1031	0.1013	0.000	0.003	**2.58**	2.519	**NA**
S1	5.000	0.1310	0.1403	0.1357	0.034	0.007	**4.85**	4.946	**98.93**
S2	10.000	0.1963	0.2010	0.1987	0.097	0.003	**1.67**	9.392	**93.92**
S3	20.000	0.3473	0.3470	0.3472	0.246	0.000	**0.06**	19.872	**99.36**
S4	40.000	0.6520	0.6672	0.6596	0.558	0.011	**1.63**	41.922	**104.81**
S5	60.000	0.9070	0.8924	0.8997	0.798	0.010	**1.15**	58.867	**98.11**

The % adsorption was calculated from the concentration of sample in supernatant with respect to concentration of same sample without centrifugation in percentage which was subtracted from 100. The percent adsorption is provided in Table-59.

Table-59: Percent adsorption calculation

Sample Type	Sample Name	Dilution Factor	Abs @ 562 nm_Read 1	Abs @ 562 nm_Read 2	Normalized Absorbance	Conc. Protein	Conc. (µg/mL)	Std Dev	CV %	% Adsorption
Sample without centrifugation	**090224-F3**	12	0.7068	0.7158	0.607	43.798	**525.6**	0.01	0.00	**90.8**
Soluble Form	**090224-F3**	1	0.7846	0.7805	0.678	48.604	**48.6**	0.00	0.01	
Pellet	**090224-F3**	12	0.6394	0.6439	0.537	39.100	469.2	0.00	0.00	

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WE CLAIM:

1. A modified factor H binding protein (fHbp) comprising wild type fHbp variant and at least one exogenous loop(s), wherein
    - the modified factor H binding protein (fHbp) is selected from amino acid sequence with at least 75% identity with any one of sequences of SEQ ID Nos 6 to 10,
    - the at least one exogenous peptide loop(s) is immunogenic,
    - the at least one exogenous peptide loop(s) is derived from a bacterial membrane protein,
    - the modified fHbp is a fusion protein,
    - the fHbp variant is selected from v1, v2 and v3, modified with at least one PorA loop comprising at least 10 amino acids inserted into a β-turn region in fHbp; and
    - the PorA loop is selected from VR1, and VR2.

2. The modified factor H binding protein as claimed in claim 1, wherein the modified fHbp is modified to reduce factor H binding activity.

3. A nucleic acid sequence encoding the modified fHbp, wherein the nucleic acid sequence is with at least 75% identity with any one of sequences of SEQ ID NO. 1 to 5.

4. An immunogenic composition comprising at least one modified fHbp as claimed in any one of the claims 1 to 2 or the nucleic acid sequence encoding the modified fHbp as claimed in claim 3.

5. The immunogenic composition as claimed in claim 4, wherein the composition comprises two or more different modified fHbp.

6. The immunogenic composition as claimed in any one of the claims 4 to 5, wherein the composition comprises a pharmaceutically acceptable carrier.

7. The immunogenic composition as claimed in any one of the claims 4 to 6, wherein the composition further comprises an adjuvant.

8. The immunogenic composition as claimed in any one of the claims 4 to 7, wherein the composition further comprises at least one other prophylactically or therapeutically active molecule comprising a monovalent carrier protein: capsule polysaccharide conjugate vaccine.

9. The immunogenic composition as claimed in any one of the claims 4-8, wherein the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the conjugate vaccine.

10. The immunogenic composition as claimed in any one of the claims 4 to 9 comprising a recombinant protein/ modified fHbp fusion protein in combination with at least one additional antigen selected from:

- a protein antigen from PorB, Fet A, OmpC, NHBA, NadA, meningococcal antigen 287, NspA, HmbR, NhhA, App, 936,
- a saccharide or conjugate antigen from N. meningitidis serogroup A, C, W, Y and/or X,
- a saccharide or conjugate antigen from Streptococcus pneumoniae,
- a diphtheria antigen, such as a diphtheria toxoid e.g. the CRM197 mutant,
- a tetanus antigen, such as a tetanus toxoid,
- an antigen from Bordetella pertussis, acellular or whole cell pertussis antigens,
- a saccharide or conjugate antigen from Haemophilus influenzae B,
- polio antigen(s) such as IPV,
- measles, mumps and/or rubella antigens,
- influenza antigen(s), such as the haemagglutinin and/or neuraminidase surface proteins,

- antigen (protein or saccharide or conjugate) from Streptococcus agalactiae (group B streptococcus),

- antigen (protein or saccharide or conjugate) from Streptococcus pyogenes (group A streptococcus),

- antigen (protein or saccharide or conjugate) from Staphylococcus aureus,

- antigen (protein or saccharide or conjugate) from Salmonella Spp.

11. The immunogenic composition as claimed in any one of the claims 4 to 10, wherein the fHbp scaffold bearing exogenous peptide loops is incorporated as the protein carrier molecule in the polysaccharide conjugate vaccine selected from Monovalent (A, X, C, W, Y), Bivalent (A-C, A-B, X-B, C-B), Trivalent (AC-B, AC-Hib), Quadrivalent (AC-Hib-B), Pentavalent (ACWYX), or Hexavalent (ACWYX-B).

12. The modified fHbp as claimed in any one of claims 1 to 2, a nucleic acid as claimed in claim 3, or a composition as claimed in any one of claims 4 to 8, for use as a medicament, or in treatment or prevention of a pathogenic infection or colonization in a subject.

13. A combination of the modified fHbp as claimed in any one of claims 1 to 2, a nucleic acid as claimed in claim 3, or a composition as claimed in any one of claims 4 to 8, and at least one other prophylactically or therapeutically active molecule.

14. The combination as claimed in claim 13 or composition as claimed in claim 8, wherein the at least one other prophylactically or therapeutically active molecule comprises a conjugate vaccine, comprising any of serogroup capsular polysaccharides selected from A, C, Y, W, or X strains, or combinations thereof.

15. The combination as claimed in claim 14 or the composition as claimed in claim 8, wherein the protein: capsule polysaccharide vaccine comprises any of serogroup C or A capsule with bacterial toxoids, bivalent vaccines (with serogroup C and A capsular polysaccharide conjugated to bacterial toxoids), quadrivalent (serogroups A, C, Y, W

polysaccharides conjugated to bacterial toxoids) or pentavalent (serogroups A, C, Y, W, X polysaccharides conjugated to bacterial toxoid) conjugate vaccines.

16. The factor H binding protein (fHbp) as claimed in claims 1 to 2 as and when used as an epitope display scaffold.

17. A vaccine formulation comprising at least one recombinant protein/ modified fHbp fusion protein, an adjuvant and one or more pharmaceutically acceptable excipient, wherein in the recombinant protein/ modified fHbp fusion protein the one or more exogenous loops is selected from but not limited to group consisting of Transferrin Binding Protein, Neisserial Heparin Binding Protein, Neisserial Surface Protein A, PorA, meningococcal enterobactin receptor FetA, Neisserial Adhesin A, the fHbp-fHbp fusion protein as claimed in any one of the previous claims or a combination thereof.

18. The vaccine formulation as claimed in claim 17, wherein the recombinant protein/ modified fHbp fusion protein is fHbp as claimed in any one of claims 1-2, or the nucleic acid sequence encoding the modified fHbp as claimed in claim 3, and wherein the fHbp is derived from Neisseria meningitidis serogroup A, B, C, H, I, K, L, 29E, W135, X, Y and Z.

19. The vaccine formulation as claimed in claims 17 or 18, wherein the recombinant protein/ modified fHbp fusion protein is derived from Neisseria meningitidis serogroup B.

20. The vaccine formulation as claimed in any one of the claims 17-19, wherein the recombinant protein/ modified fHbp fusion protein is fHbp has molecular weight in the range of 10 kDa to 200 kDa, preferably up to 50 kDa.

21. The vaccine formulation as claimed in any one of the claims 17-20, wherein the adjuvant is selected from the group consisting of aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate, MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, GLA, 3–deacylated monophosphoryl lipid A, AS01, AS03,

AF3, IL-2, RANTES, GM- CSF, TNF-a, IFN-g, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL, an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers, polymer p 1005, CRL-8300 adjuvant, muramyl dipeptide, such as an agonist of TLR1/2 TLR2, TLR3, TLR-4 agonists, TLR5, TLR7, TLR7/8, TLR8, TLR9, ODN 2216 (type A), TLR11/12, TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, Alpha-C-galactosylceramide, Chitosan, Interleukin-2, QS-21, squalene, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, mycobacterium cell wall preparations, mycolic acid derivatives, non-ionic block copolymer surfactants, OMV, fHbp, saponin combination with sterols and lipids, dmLT, 1,25-dihydroxyvitamin D3, CAF01, poly [di (carboxylatophenoxy)- phosphazene] (PCPP) and Venezuelan equine encephalitis (VEE) replicon particles or a combination thereof.

22. The vaccine formulation as claimed in any one of the claims 17-21, wherein the adjuvant is aluminium hydroxide having particle size > 500 nm.

23. The vaccine formulation as claimed in any one of the claims 17-20, wherein the one or more pharmaceutically acceptable excipient is

a. a buffering agent selected from carbonate, phosphate, acetate, HEPES, Succinate, TRIS, borate, citrate, lactate, gluconate, tartrate, or a combination thereof;
b. a sugar selected from trehalose, mannose, raffinose, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose, dextrose, fructose, or a combination thereof;
c. a sugar alcohol or polyol selected from mannitol, lactitol, sorbitol, glycerol, xylitol, maltitol, lactitol, erythritol, isomalt and hydrogenated starch hydrolysates or a combination thereof;
d. a surfactant selected from polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 85, nonylphenoxypolyethoxethanol,

octylphenoxypolyethoxethanol, oxtoxynol 40, nonoxynol- 9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene- 660 hydroxystearate, polyoxyethylene- 35 ricinoleate, soy lecithin, a poloxamer, copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), octoxynols, phospholipids, nonylphenol ethoxylates, polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols, sorbitan esters or a combination thereof;

e. a polymer selected from dextran, carboxymethylcellulose, hyaluronic acid, cyclodextrin or a combination thereof;

f. a salt selected from NaCl, KCl, KH_2PO_4, $Na_2HPO_4.2H_2O$, $CaC1_2$, $MgC1_2$, or a combination thereof;

g. an amino acid selected from tricine, leucine, iso-leucine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Methionine, Proline, Serine, Threonine, or a combination thereof;

h. a hydrolysed protein selected from gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate, whey protein hydrolysate, serum albumin or a combination thereof;

i. a preservative selected from phenoxyethanol, Benzethonium chloride (Phemerol), Phenol, m-cresol, Thiomersal, Formaldehyde, paraben esters, benzalkonium chloride, benzyl alcohol, chlorobutanol, p-chlor-m-cresol, benzyl alcohol or a combination thereof; and

j. a liquid carrier selected from water for injection (WFI) or saline.

24. The vaccine formulation as claimed in any one of the claims 17-23, wherein the vaccine formulation comprises:

- at least one at least one recombinant protein/ at least one modified fHbp as claimed in any one of previous claims
- aluminium hydroxide;
- mannitol;
- phosphate; and
- polysorbate.

25. The vaccine formulation as claimed in any one of the claims 17-24, wherein the vaccine formulation comprises:
    - at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10; or
    - at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5; and
    - aluminium hydroxide;
    - mannitol;
    - phosphate; and
    - polysorbate.

26. The vaccine formulation as claimed in any one of the claims 17-25, comprising (i) at least one recombinant protein/ modified fHbp fusion protein; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

27. The vaccine formulation as claimed in any one of the claims 17-25, comprising (i) at least two recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

28. The vaccine formulation as claimed in any one of the claims 17-25, comprising (i) at least three recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of

0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

29. The vaccine formulation as claimed in any one of the claims 17-25, comprising (i) at least four recombinant proteins/ modified fHbp fusion proteins; (ii) aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml; (iii) mannitol in an amount in the range of 5 mg/ml to 100 mg/ml; (iv) phosphate buffer in an amount in the range of 1 mM to 10 mM; and (v) polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml; wherein each recombinant protein/ modified fHbp fusion protein is present in an amount in the range of 15 µg/ml to 150 µg/ml.

30. The vaccine formulation as claimed in any one of the claims 17-24, wherein the vaccine formulation comprises:

- at least one modified fHbp of the amino acid sequence with at least 75% identity with from any one of amino acid sequences SEQ ID NO. 6 to 10; or
- at least one modified fHbp encoded the nucleic acid sequence is with at least 75% identity with by any one of nucleic acid sequence SEQ ID NO. 1 to 5;

  and
- aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
- mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
- phosphate buffer in an amount in the range of 1 mM to 10 mM; and
- polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

31. The vaccine formulation as claimed in any one of the claims 17-30, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml; or
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml; or
c. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml; or
d. fHbpV1.1:PorA307-311/exP1.4 (of SEQ ID NO. 9 or encoded by SEQ ID NO. 4); in an amount in the range of 15 µg/ml to 150 µg/ml; and
e. aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;

f. mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

32. The vaccine formulation as claimed in any one of the claims 17-30, comprising:
a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/ml to 150 µg/ml;
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/ml to 150 µg/ml;
c. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/ml to 150 µg/ml;
d. fHbpV1.1:PorA307-311/exP1.4 (SEQ ID NO. 9 or encoded by SEQ ID NO. 4) in an amount in the range of 15 µg/ml to 150 µg/ml;
e. aluminium hydroxide in an amount in the range of 0.5 mg/ml to 4.5 mg/ml;
f. mannitol in an amount in the range of 5 mg/ml to 100 mg/ml;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/ml to 2 mg/ml.

33. The vaccine formulation as claimed in any one of the claims 17-30, comprising:
a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL; or
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL; or
c. fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL; or
d. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3) in an amount in the range of 15 µg/mL to 150 µg/mL; and
e. aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
f. mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

34. The vaccine formulation as claimed in any one of the claims 17-30, comprising:

a. fHbpV3.45 M5:PorA316-320/exP1.14 (of SEQ ID NO. 6 or encoded by SEQ ID NO. 1) in an amount in the range of 15 µg/mL to 150 µg/mL;
b. fHbpV2.19 M6:PorA316-320/exP1.4 (of SEQ ID NO. 7 or encoded by SEQ ID NO. 2) in an amount in the range of 15 µg/mL to 150 µg/mL;
c. fHbpV1.1:PorA307-311/exP1.9 (of SEQ ID NO. 10 or encoded by SEQ ID NO. 5) in an amount in the range of 15 µg/mL to 150 µg/mL;
d. fHbpV1.14:PorA307-311/exP1.9 (of SEQ ID NO. 8 or encoded by SEQ ID NO. 3)in an amount in the range of 15 µg/mL to 150 µg/mL;
e. aluminium hydroxide in an amount in the range of 0.5 mg/mL to 4.5 mg/mL;
f. mannitol in an amount in the range of 5 mg/mL to 100 mg/mL;
g. phosphate buffer in an amount in the range of 1 mM to 10 mM; and
h. polysorbate 20 in an amount in the range of 0.01 mg/mL to 2 mg/mL.

35. The vaccine formulation as claimed in any one of the claims 17-34, wherein the formulation comprises 2-phenoxyethanol in an amount in range of 1 mg/mL to 10 mg/ml.

36. The vaccine formulation as claimed in any one of the claims 17-35, wherein the vaccine composition is stable at 2-8°C, 25°C and 40°C for over a period of six months.

37. The vaccine formulation as claimed in any one of the claims 17-35, having Zeta potential in the range of -16 mV to -30 mV; osmolality in the range of 200 mOsmol/kg to 500 mOsmol/kg.

38. The vaccine formulation as claimed in any one of the claims 17-35, further comprising one or more antigen selected from Diphtheria toxoid (D), Tetanus toxoid (T), Whole cell pertussis (wP), hepatitis B virus surface antigen (HbsAg), Haemophilus influenzae b PRP-Carrier protein conjugate (Hib), Haemophilus influenzae (a, c, d, e, f serotypes and the unencapsulated strains), Neisseria meningitidis A antigen(s), Neisseria meningitidis C antigen(s), Neisseria meningitidis W-135 antigen(s), Neisseria meningitidis Y antigen(s), Neisseria meningitidis X antigen(s), Streptococcus Pneumoniae antigen(s), Neisseria meningitidis B bleb or purified antigen(s), Staphylococcus aureus antigen(s), Anthrax, BCG, Hepatitis (A, C,

D, E, F and G strains) antigen(s), Human papilloma virus, HIV, Salmonella typhi antigen(s), acellular pertussis, modified adenylate cyclase, Malaria Antigen (RTS,S), Measles, Mumps, Rubella, Dengue, Zika, Ebola, Chikungunya, Japanese encephalitis, rotavirus, Diarrheal antigens, Flavivirus, smallpox, yellow fever, Shingles, Varicella virus antigens, and combinations thereof.

39. The vaccine formulation as claimed in any one of the claims 17-35, 38 comprising (i) at least one fusion protein comprising stable non-functional/ non-lipidated fHbp and PorA VR2 loop, and (ii) at least one polysaccharide-protein conjugate.

40. The vaccine formulation as claimed in any one of the claims 17-39, wherein the recombinant protein/ modified fHbp fusion protein is co-administered with one or more vaccine selected from BEXSERO, MENVEO, MENACTRA, NIMENRIX, MenQuadFi, MENFIVE, MenAfriVac, Men AC, and Men ACHib.

41. The vaccine formulation as claimed in any one of the claims 17-40, wherein the recombinant protein/ modified fHbp fusion protein is co-administered with MENFIVE.

42. The vaccine formulation as claimed in any one of the claims 17-41 comprising i) at least one fusion protein comprising stable non-functional /non-lipidated fHbp and PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

43. The vaccine formulation as claimed in any one of the claims 17-41 comprising comprises i) at least two fusion proteins, each consisting of one fHbp variant type coupled to one PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N.

meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

44. The vaccine formulation as claimed in any one of the claims 17-41 comprising i) at least three fusion proteins, each consisting of one fHbp variant type coupled to two PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

45. The vaccine formulation as claimed in any one of the claims 17-41 comprising i) at least four fusion proteins, each consisting of one fHbp variant type coupled to three PorA VR2 loop and at least one conjugate selected from (a) a conjugate of (i) the capsular saccharide of serogroup A N. meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N. meningitidis and (ii) CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197; (d) a conjugate of (i) capsular saccharide of serogroup W135 N. meningitidis and (ii) CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N. meningitidis and (ii) tetanus toxoid.

46. The vaccine formulation as claimed in any one of the claims 17-45 for use in the treatment or prevention of infection and/or disease caused by Neisseria meningitidis serogroup B.

47. The vaccine formulation as claimed in any one of the claims 17-45, wherein the vaccine formulation elucidates cross protection against Neisseria gonorrhea strains and Neisseria meningitidis serogroups ACWYX.

48. The vaccine formulation as claimed in any one of the claims 17-45, wherein the percent adsorption of the recombinant protein/ modified fHbp fusion protein on to an adjuvant is in the range of 70 % to 100 %.

49. The vaccine formulation as claimed in claim 48, wherein percent adsorption of fHbp V3.45 M5 PorA $^{316\text{-}320\ exP1.14}$ on to an adjuvant is in the range of 80 % to 100 %.

50. The vaccine formulation as claimed in claim 48, wherein the percent adsorption of fHbp V1.14 PorA $^{307\text{-}311\ exP1.9}$ on to an adjuvant is in the range of 80 % to 90 %.

51. The vaccine formulation as claimed in claim 48, wherein the percent adsorption of fHbp V2.19 PorA $^{316\text{-}320\ exP1.4}$ on to an adjuvant is in the range of 80 % to 90 %.

52. The vaccine formulation as claimed in claim 48, wherein the percent adsorption of fHbp V1.1 PorA $^{307\text{-}311\ exP1.4}$ on to an adjuvant is in the range of 80 % to 90 %.

53. The vaccine formulation as claimed in claim 48, wherein the percent adsorption of fHbp V1.1 PorA $^{307\text{-}311\ exP1.9}$ on to an adjuvant is in the range of 70 % to 80 %.

54. A method for manufacturing a vaccine formulation as claimed in any one of the claims 17-53, the method comprising the following steps:

(a) growing host cells comprising the expression vector in nutrient medium;
(b) inducing the host cells for expressing protein;
(c) harvesting and separating the host cells;
(d) lysing the harvested cells and separating host cell debris to obtain tagged proteins;
(e) purifying the tagged proteins;
(f) removing tags from the tagged proteins to obtain recombinant proteins/ modified fHbp fusion proteins;
(g) purifying the recombinant proteins/ modified fHbp fusion proteins; and
(h) preparing the vaccine formulation comprising the purified recombinant proteins/ modified fHbp fusion proteins.

55. The method as claimed in claim 54, wherein the host cell is a bacterial expression host system.

56. The method as claimed in claim 55, wherein the bacterial expression host system is an Escherichia coli strain selected from the group consisting of BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**, Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3) or B834(DE3).

57. The method as claimed in claim 54, wherein the nutrient medium is selected from Undefined medium, Terrific Broth (TB) Medium, Lysogenia Broth, Luria Broth or Luria-Bertani medium, chemically defined medium, M9 Minimal Medium, Chemically Defined M9 Modified Salt Medium, 2xYT medium or Super Optimal broth with Catabolite repression (SOC) Medium and combinations thereof.

58. The method as claimed in claim 54, wherein the concentration of L-methionine during growth of host cells in step (a) is maintained in the range of 1 mM to 10 mM, wherein the fermentation is in fed batch mode.

59. The method as claimed in any claim 54, wherein the host cells are grown at a temperature in the range of 35°C to 39°C; pH in the range of 5.0 to 9.0; dissolved oxygen in the range of 10 to 100%; agitation in the range of 100–1800 rpm; gas flow rate in the range of 0-2 volume of gas per unit volume of liquid per minute (VVM).

60. The method as claimed in claim 54, wherein the host cells are induced using an inducer selected from lactose and its non-hydrolyzable analog isopropyl β-D-1-thiogalactopyranoside (IPTG).

61. The method as claimed in claim 60, wherein the concentration of lactose is in the range of 1 g/L to 50 g/L and the concentration of IPTG is in the range of 1 mM to 10 mM.

62. The method as claimed in claim 54, wherein the host cells are lysed using a method selected from chemical mode, biological mode, physical mode, mechanical mode, and a combination thereof.

63. The method as claimed in claim 62, wherein the host cells are lysed using a combination of chemical and mechanical mode.

64. The method as claimed in any one of the claims 62-63, wherein the host cells are lysed using a lysis buffer having a pH in the range of 7 to 9, followed by mechanical lysis at a pressure in the range of 1000-1500 Bar for 3 to 8 cycles.

65. The method as claimed in claim 64, wherein the mechanical lysis is carried out using a homogenizer.

66. The method as claimed in claim 54, wherein the tagged protein in step (e) is purified using chromatography step, followed by concentration and diafiltration.

67. The method as claimed in claim 54, wherein the tags are removed using TEV protease having a protein: TEV protease ratio in the range of 5:1 to 30:1.

68. The method as claimed in claim 54, wherein the recombinant protein/ modified fHbp fusion protein in step (g) is purified using chromatography step, followed by concentration and diafiltration.

69. The method as claimed in claimed in claim 68, wherein the chromatography is selected from column chromatography, ion-exchange chromatography, anion exchange chromatography, cation exchange chromatography, column chromatography, flash chromatography, gel filtration/ size-exclusion/ gel-permeation (molecular sieve) chromatography, affinity chromatography, paper chromatography, thin-layer chromatography, gas chromatography, dye-ligand chromatography, hydrophobic interaction chromatography, pseudoaffinity chromatography, liquid chromatography, high-pressure liquid chromatography (HPLC), immobilized metal affinity chromatography, anion exchange chromatography, cation exchange

chromatography, multimodal chromatography, multimodal anion exchange chromatography, electrostatic interaction chromatography, hydrogen bonding chromatography, reverse phase chromatography, and combinations thereof

70. The method as claimed in any one of the claims 54-69, wherein the tagged proteins are expressed as inclusion bodies (IB) and are purified by urea unfolding of protein and on column refolding of the protein.

71. The method as claimed in any claim 54, wherein the vaccine formulation is prepared by adsorbing individual recombinant protein/ modified fHbp fusion protein on to an adjuvant, followed by addition to an excipient mixture comprising a sugar alcohol, a buffering agent, a stabilizer, and a liquid carrier.

72. The method as claimed in claim 71, wherein the excipient mixture comprises a preservative.

73. The method as claimed in claim 67, wherein TEV protease is produced by a method comprising the following steps:

(a) growing host cells comprising the expression vector in nutrient medium;
(b) inducing the host cells for expressing TEV protease;
(c) harvesting and separating the host cells;
(d) lysing the harvested and separating host cells to obtain TEV protease;
(e) purifying the TEV protease; and
(f) concentrating, diafiltration and storing the purified TEV protease.

74. The method as claimed in claim 73, wherein the host cell for expressing TEV protease is an Escherichia coli strain selected from the group consisting of BL21 (DE3), BL21 (DE3) pLysS*, BL21 (DE3) pLysE*, BL21 star (DE3), BL21-A1, BLR (DE3), HMS174 (DE3)**, Tuner (DE3), Origami2 (DE3)**, Rosetta2 (DE3)*, Rosettagami (DE3), Lemo21 (DE3)*, T7 Express, m15 pREP4*, C41(DE3), C43(DE3), Rosetta™(DE3)pLysS or B834(DE3).

75. The method as claimed in any one of the claims 54-74, wherein the glucose feed is stopped, and glycerol feed is started when OD at 590/ 600 nm is 20-100 and inducing the culture by adding and/ or maintaining lactose at 1-50 g/L in fed batch mode.

76. A method for inducing an immune response against Neisseria meningitidis serogroup B strain in an individual by administering to the individual a vaccine formulation as claimed in any one of the previous claims, wherein the step of administration induces an immune response against the Neisseria meningitidis serogroup B strain.

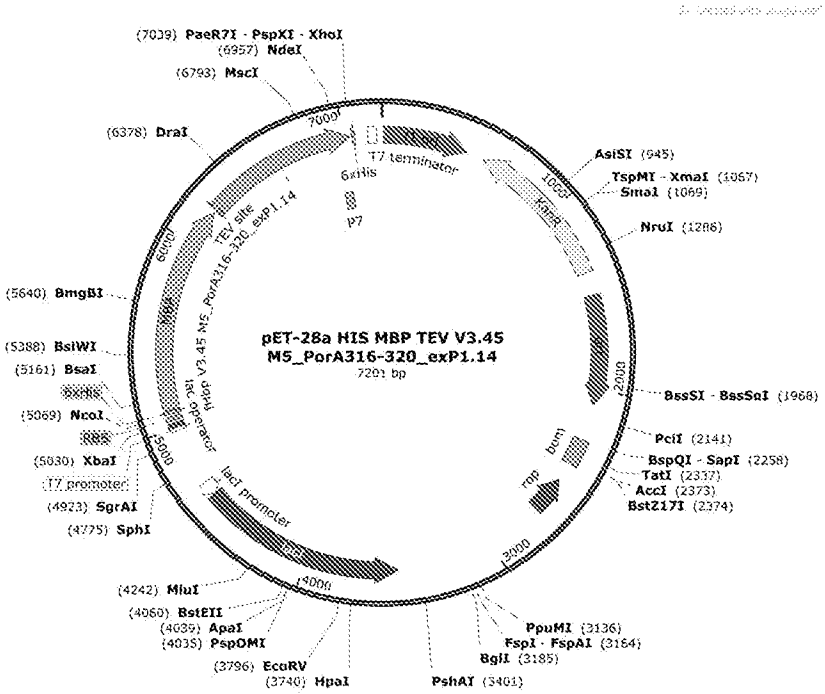

Figure-1

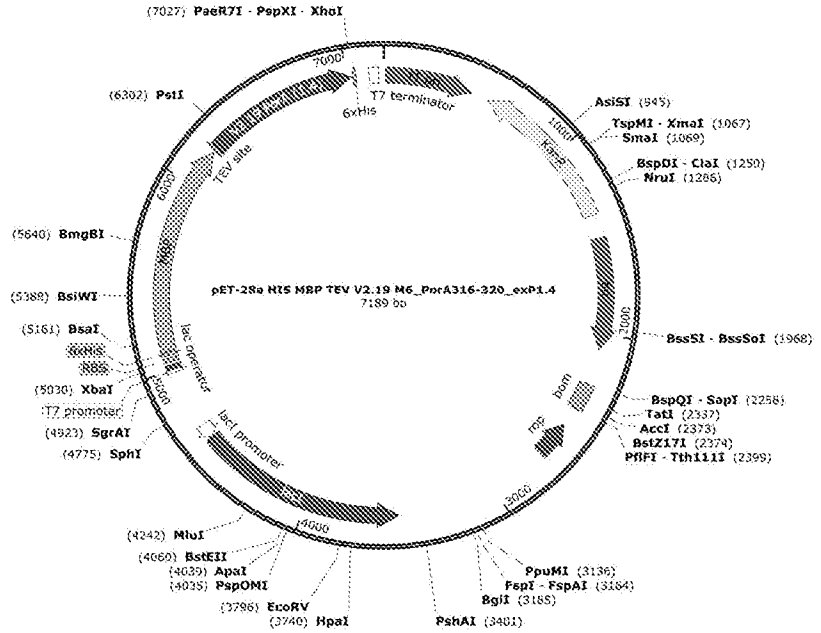

Figure-2

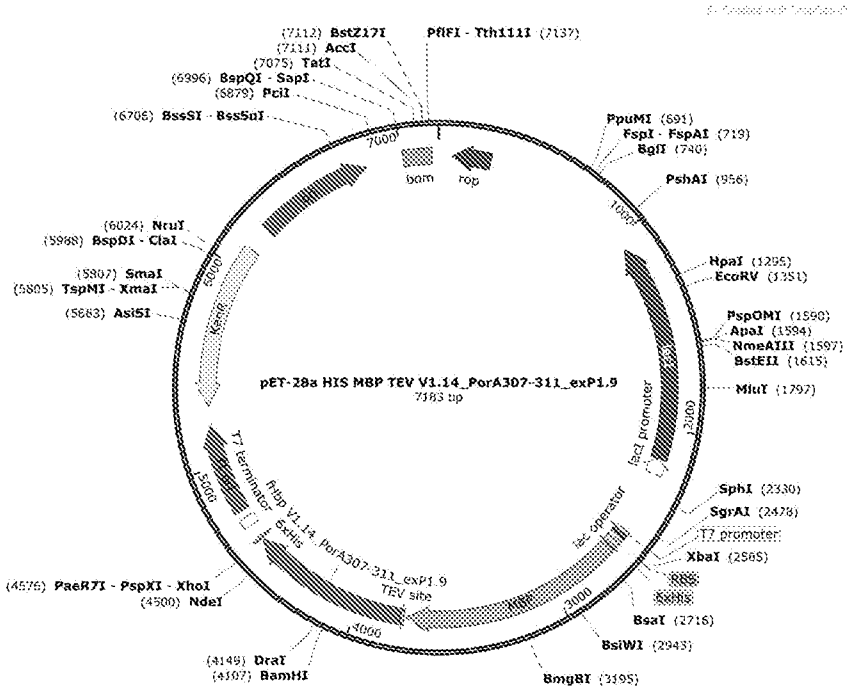

Figure-3

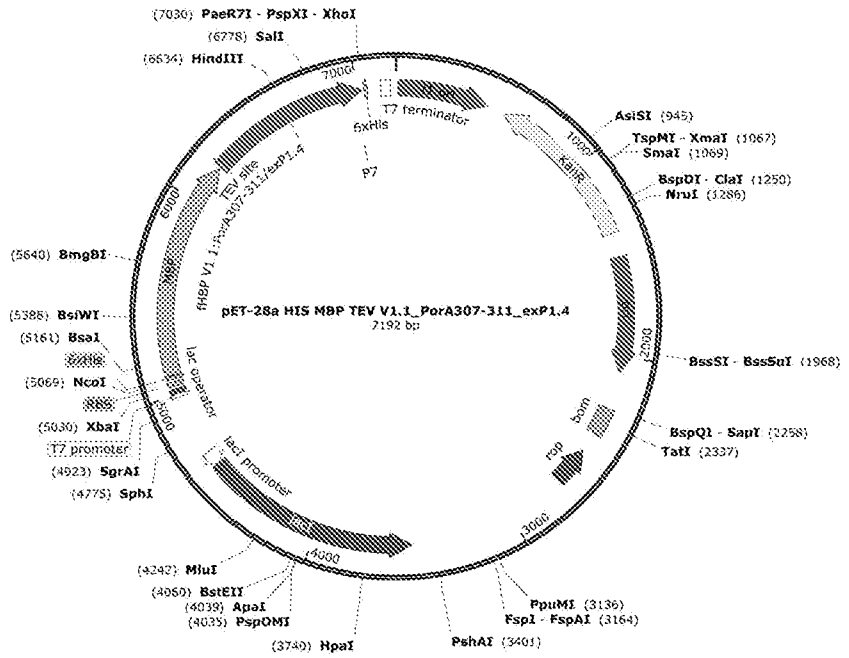

Figure-4

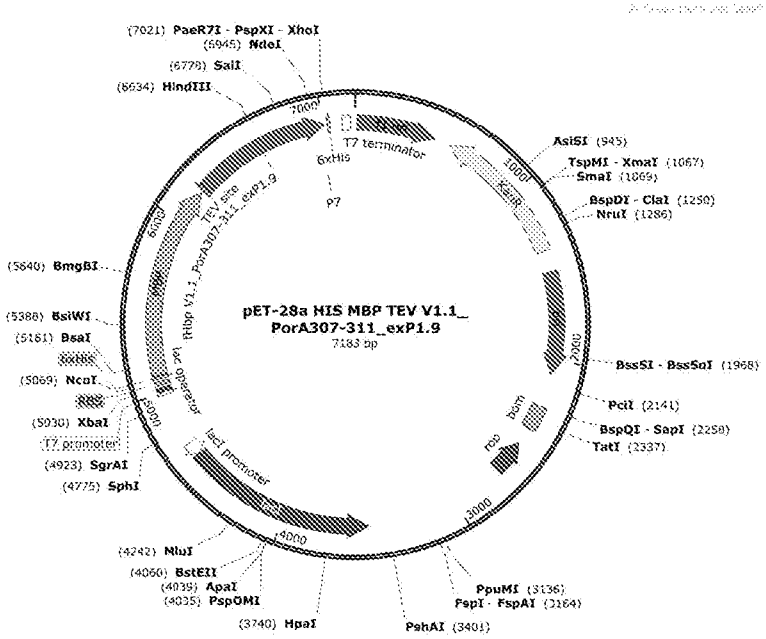

Figure-5

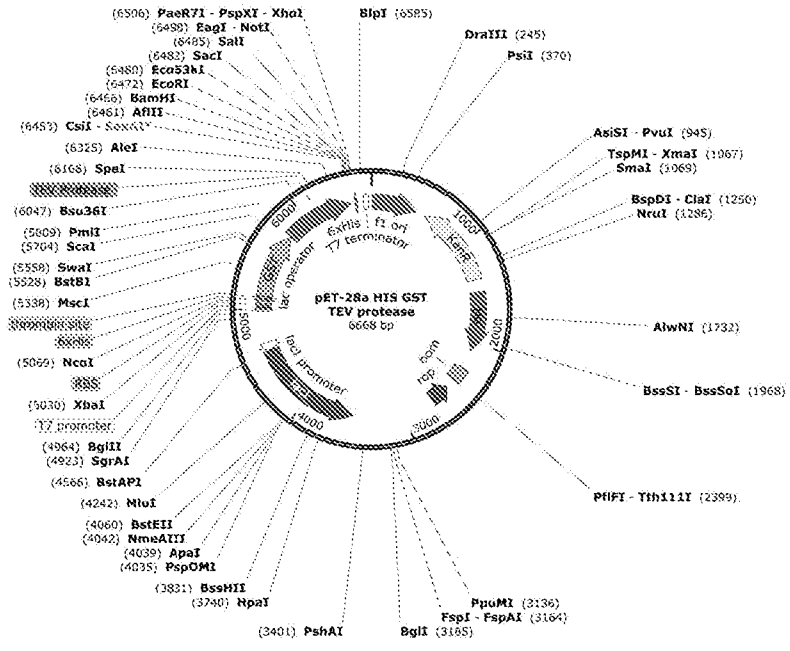

Figure-6

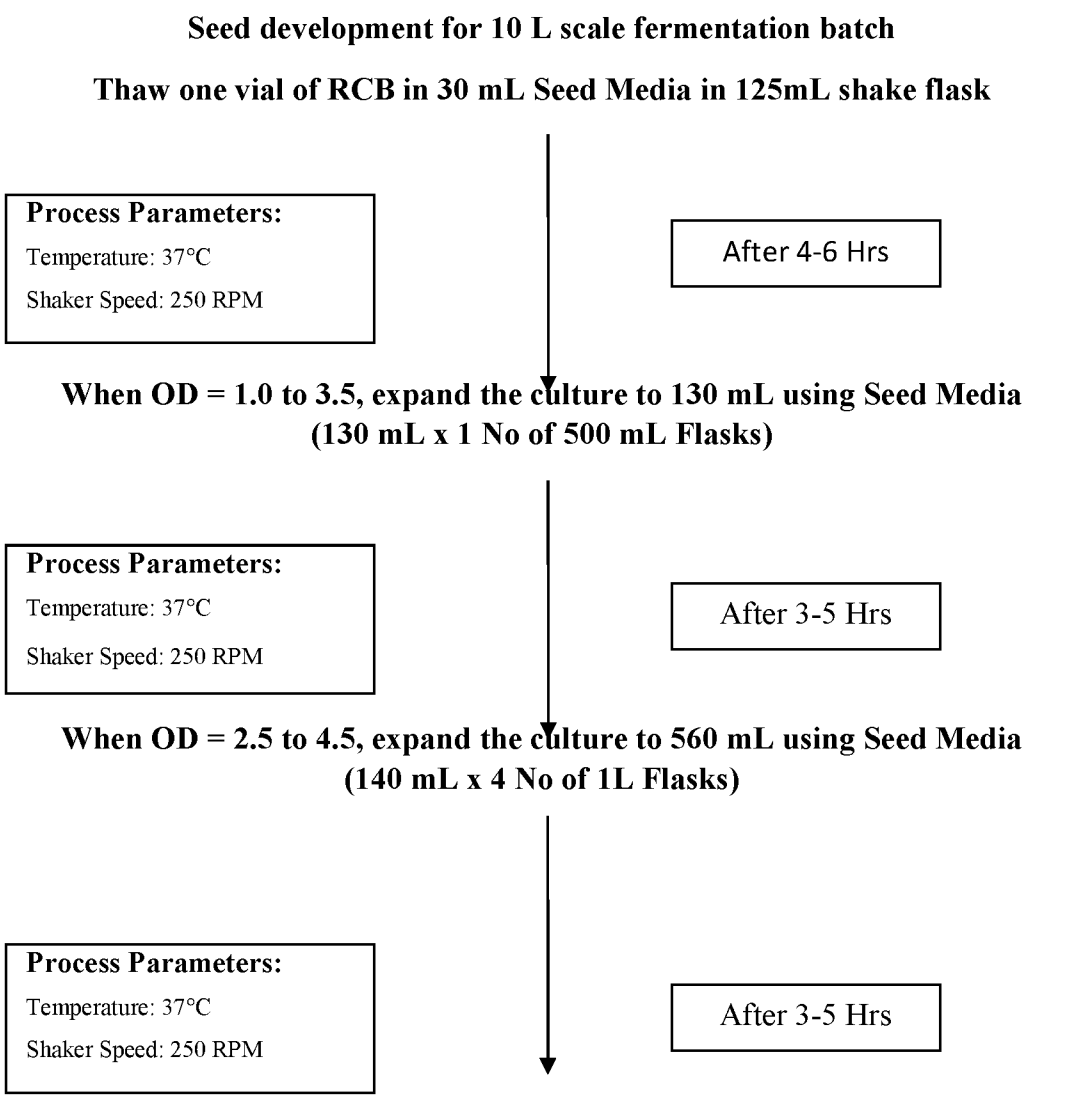
Seed development for 10 L scale fermentation batch
Thaw one vial of RCB in 30 mL Seed Media in 125mL shake flask
Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM
After 4-6 Hrs
When OD = 1.0 to 3.5, expand the culture to 130 mL using Seed Media
(130 mL x 1 No of 500 mL Flasks)
Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM
After 3-5 Hrs
When OD = 2.5 to 4.5, expand the culture to 560 mL using Seed Media
(140 mL x 4 No of 1L Flasks)
Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM
After 3-5 Hrs
When OD = 3.5 to 5.0, use the seed culture for inoculation of 10 L fermenter

Figure-7

Production of tagged protein at 10L scale fermenter

Inoculation: Seed Culture 550 mL + Fermentation Media 5500 mL in 10L Fermenter

(During process maintain the L-Methionine concentration to 1-10 mM)

Process Parameters:
Temp.:37°C
pH: 7.0
DO: 30%
Gas Flow: 2-5 SLPM
Agitation: 300-1500 rpm
Oxygen % in total gas flow: 0-50%

Sampling interval: every Hr. check OD@ 590nm

After 4-6 Hours

When glucose in the fermentation media gets depleted completely, start glucose feed

After 7-10 Hours

When OD at 590 nm is~ 60-100 stop glucose feed and start glycerol feed. Induce the culture by adding lactose solution in to the culture (5-20 g/L final concentration). (At induction of tagged fHbp protein fHbp V3.45 M5: PorA 316-320/exP1.14 and fHbp V2.19 M6: PorA 316-320/exP1.4 shift the temperature set point of fermenter from 37°C to 33°C)

After 4-6 Hours of Induction

Harvest the Batch
(OD at 590 nm is~ 90-180)

For cell separation, centrifuge the harvest broth at 4700 RPM (RCF= 7340) for 40 min at 4°C (Wet cell mass obtained ~ 150-350 g/L of harvest broth)

Handover Cell Pellet to DSP for protein purification

Figure-8

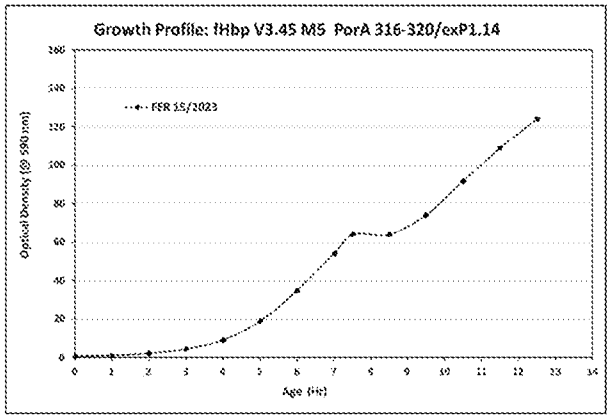

Figure-9a

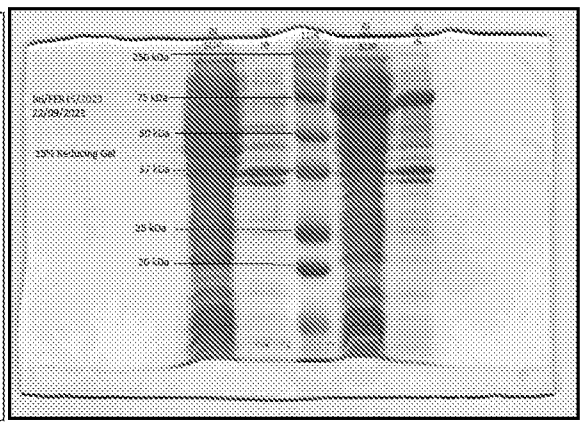

Figure-9b

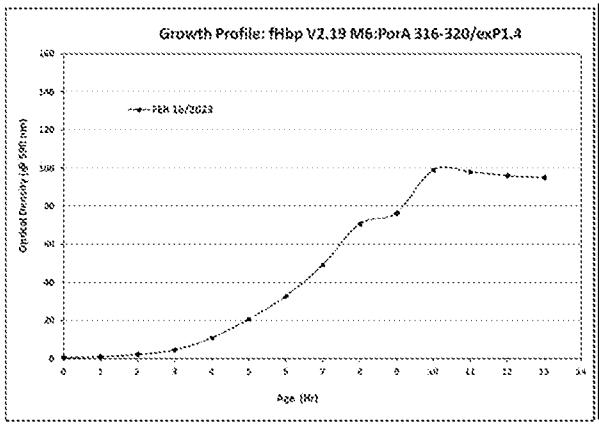

Figure-9c

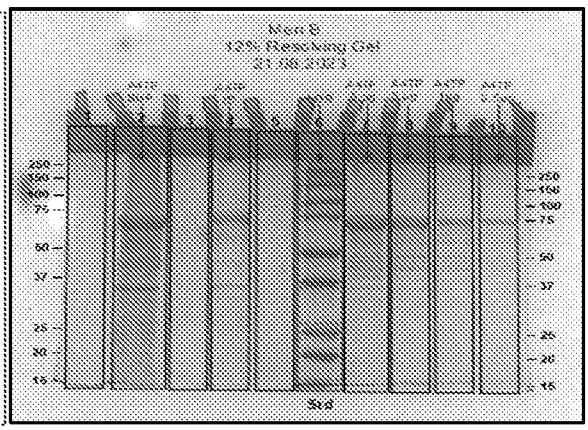

Figure-9d

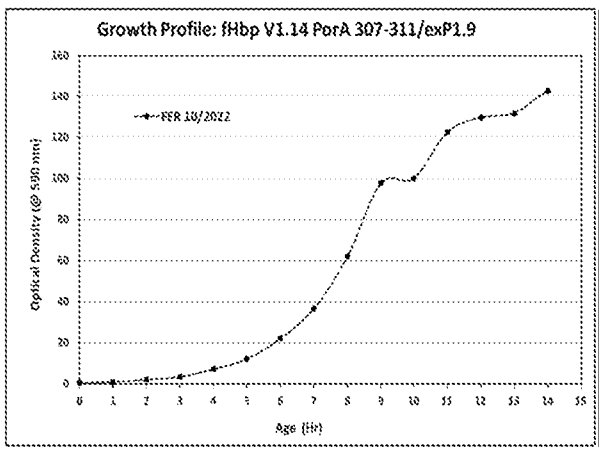

Figure-9e

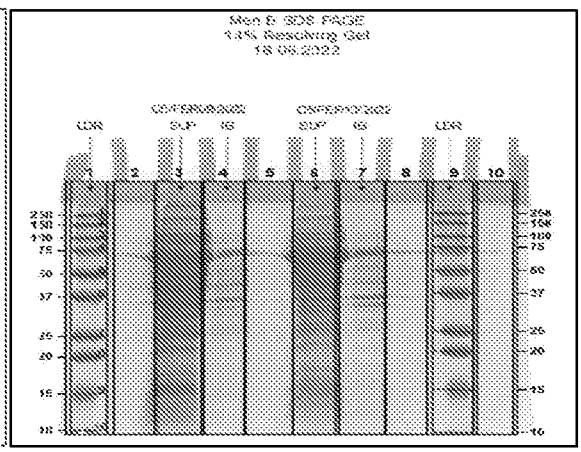

Figure-9f

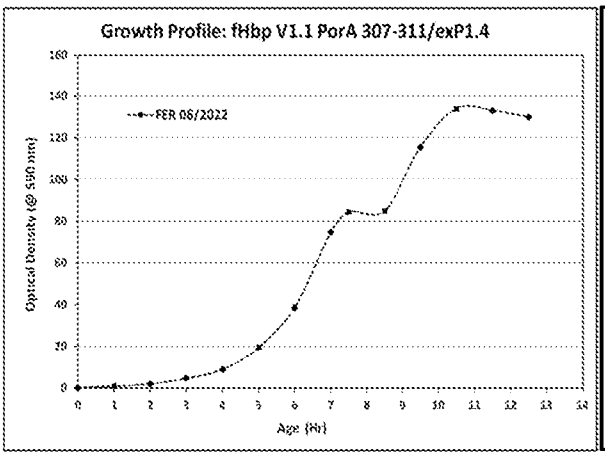

Figure-9g

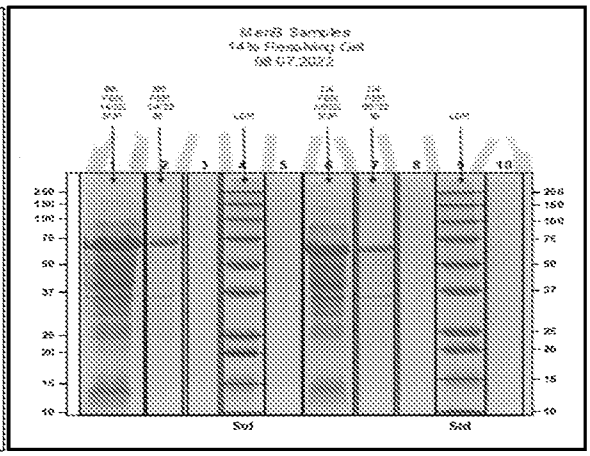

Figure-9h

Seed development for 10 L scale fermentation batch for the production of TEV protease

Thaw one vial of RCB in 30 mL Seed Media in 125mL shake flask

Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM

After 4-6 Hrs

When OD = 1.0 to 3.5, expand the culture to 130 mL using Seed Media (130 mL x 1 No of 500 mL Flasks)

Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM

After 3-5 Hrs

When OD = 2.5 to 4.5, expand the culture to 420 mL using Seed Media (140 mL x 3 No of 1L Flasks)

Process Parameters:
Temperature: 37°C
Shaker Speed: 250 RPM

After 3-5 Hrs

When OD = 3.5 to 5.0, use the seed culture for inoculation of 10 L fermenter

Figure-10

Production of TEV protease at 10 L scale fermenter

Inoculation: Seed Culture 420 mL + Fermentation Media 3600 mL in 10L Fermenter

(During process maintain the L-Methionine concentration to 1-10 mM)

Process Parameters:
Temp.:37°C
pH: 7.0
DO: 30%
Gas Flow: 2-5 SLPM
Agitation: 300-1500 rpm
Oxygen % in total gas flow: 0-50%

Sampling interval: Every Hr. Check OD@ 590nm

After 4-6 Hours

When glucose in the fermentation media gets depleted completely, start glucose feed

After 7-10 Hours

When OD at 590 nm is~ 60-80 induce the culture by adding IPTG solution in to the culture (1-5 mM final concentration). At induction, shift the temperature set point of fermenter from 37°C to 20°C

After 12-16 Hours of Induction

Harvest the Batch
(OD at 590 nm is~ 100-180)

For cell separation, centrifuge the harvest broth at 4700 RPM (RCF= 7340) for 40 min at 4°C (Wet cell mass obtained ~ 150-350 g/L of harvest broth)

Handover Cell Pellet to DSP for protein purification

Figure-11

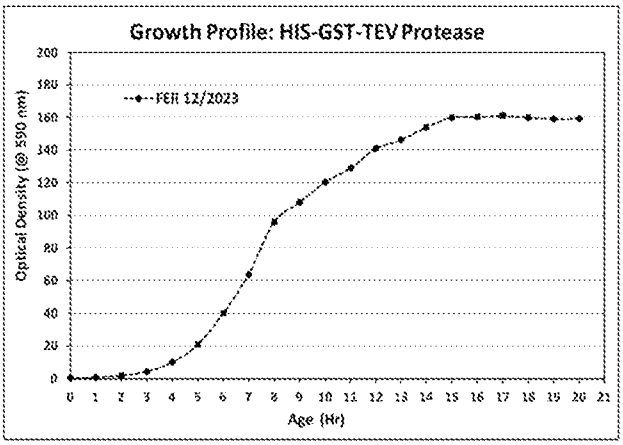

Figure-12a

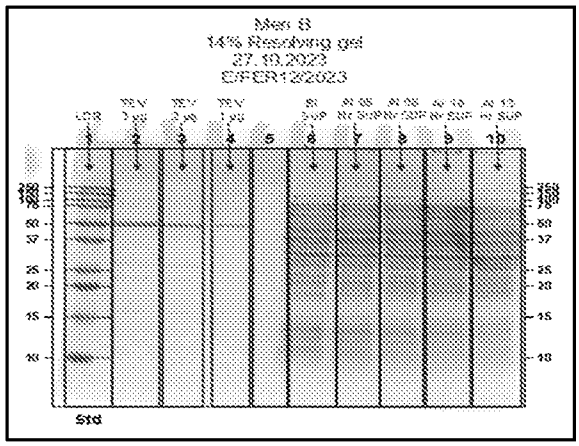

Figure-12b

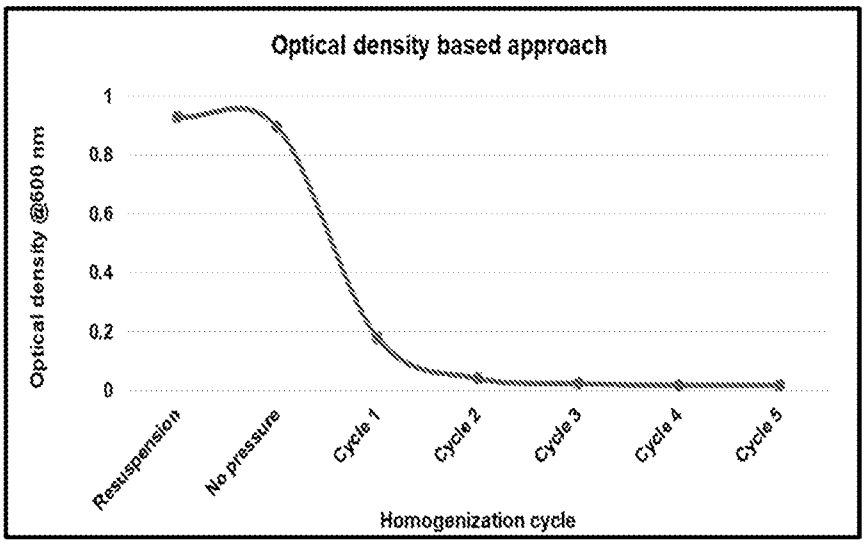

Figure-13

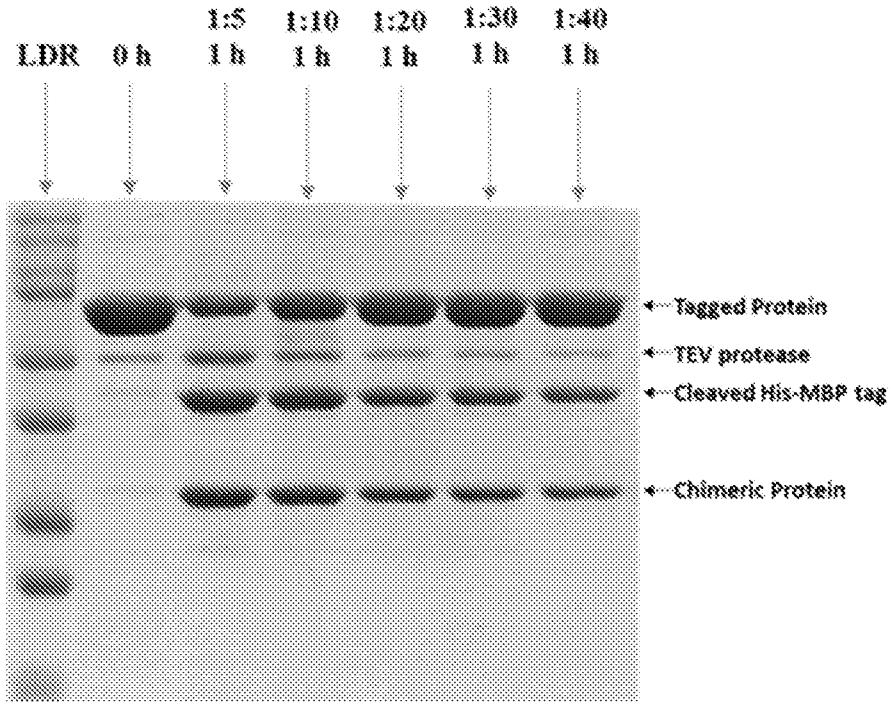

Figure-14a

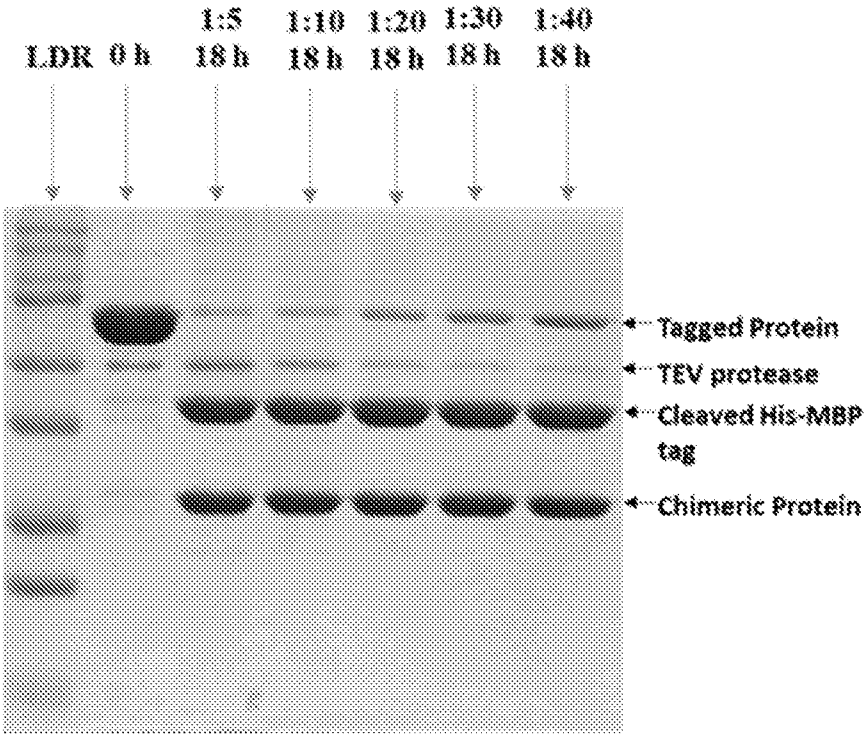

Figure-14b

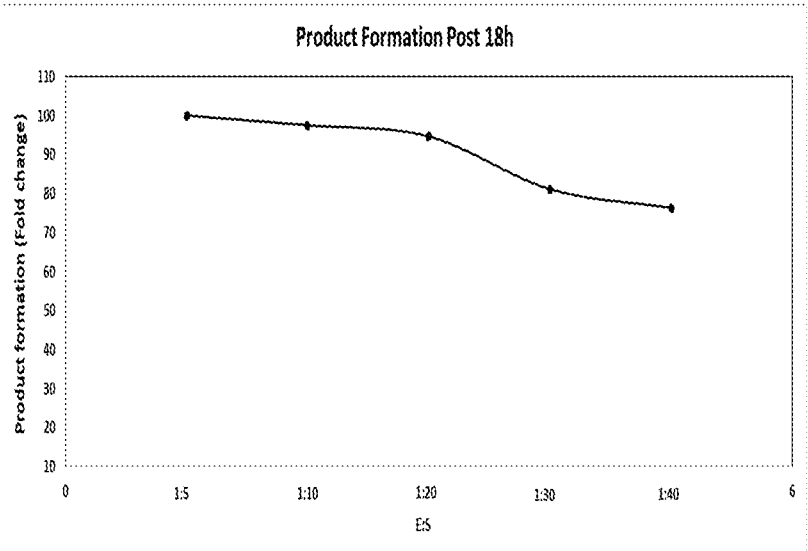

Figure-15

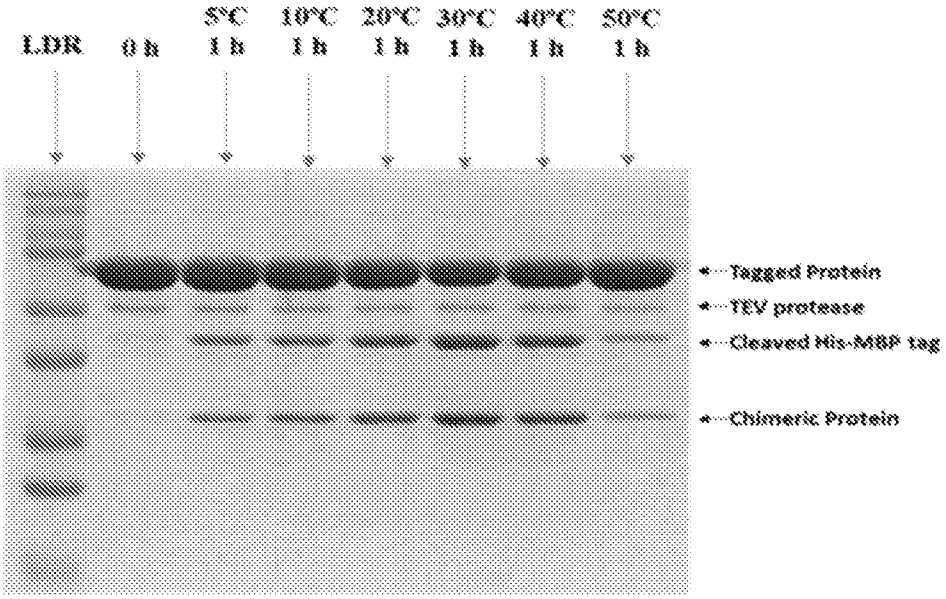

Figure-16

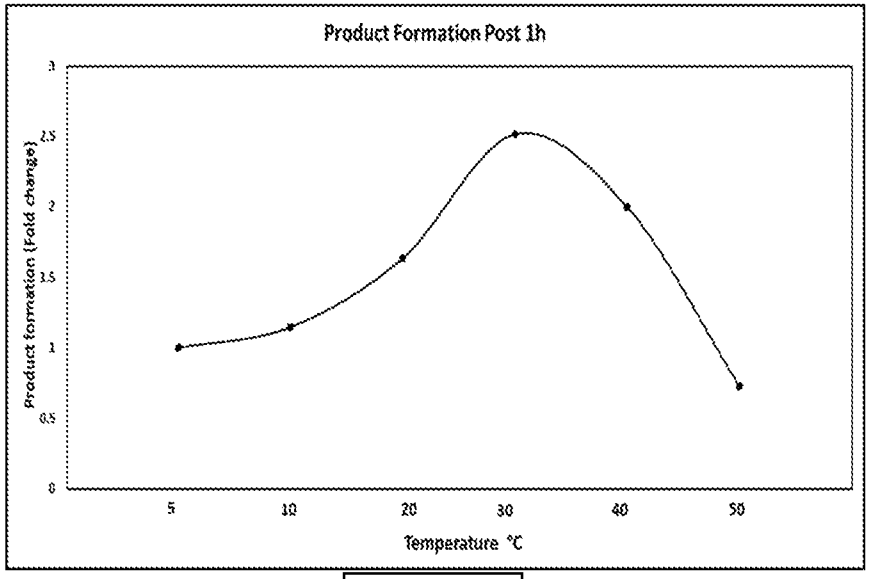
Product Formation Post 1h
Product formation (Fold change)
3
2.5
2
1.5
1
0.5
0
5
10
20
30
40
50
Temperature °C

Figure-17

Purification of TEV protease by Ni-Sepharose Resin (pH 7.4)

Cell Lysis

Harvested cell pellet 100 gram dissolved in ~2-3 L lysis buffer (25 mM Na-Phosphate buffer+100mM NaCl + 20mM Imidazol, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and up to 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

↓

Ni-Sepharose 6FF Column

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis supernatant loaded on the column

↓

Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl + 20mM Imidazol, pH 7.4); 5-6 CV

↓

Wash 2 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV

↓

Wash 3 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV

↓

Wash4 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 120mM Imidazol); 5-6 CV

↓

Elution with Buffer: (50mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)

↓

After elution purified TEV protease concentrated and diafiltration carried out with 25mM Na-Phosphate buffer pH7.4 using 10kDa cassette and stored below -20°C with 20% glycerol until further use

Figure-18

Purification of TEV protease by Ni-Sepharose Resin (pH 8.5)

Cell Lysis

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Tris-HCl buffer+100mM NaCl + 40mM Imidazol, pH 8.5) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and upto 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

↓

Ni-Sepharose 6FF Column

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis supernatant loaded on the column

↓

Wash 1 with Buffer: (25 mM Tris-HCl buffer+100mM NaCl + 40mM Imidazol, pH 8.5); 5-6 CV

↓

Wash 2 with Buffer: (25 mM Tris-HCl buffer +100mM NaCl, pH 8.5+ 100mM Imidazol); 5-6 CV

↓

Elution with Buffer:(25 mM Tris-HCl buffer +100mM NaCl, pH 8.5+ 300 mM Imidazol)

After elution purified TEV protease concentrated and diafiltration carried out with 25mM Tris-HCl buffer pH 8.5 using 10kDa cassette and stored below -20°C with 20% glycerol until further use

Figure-19

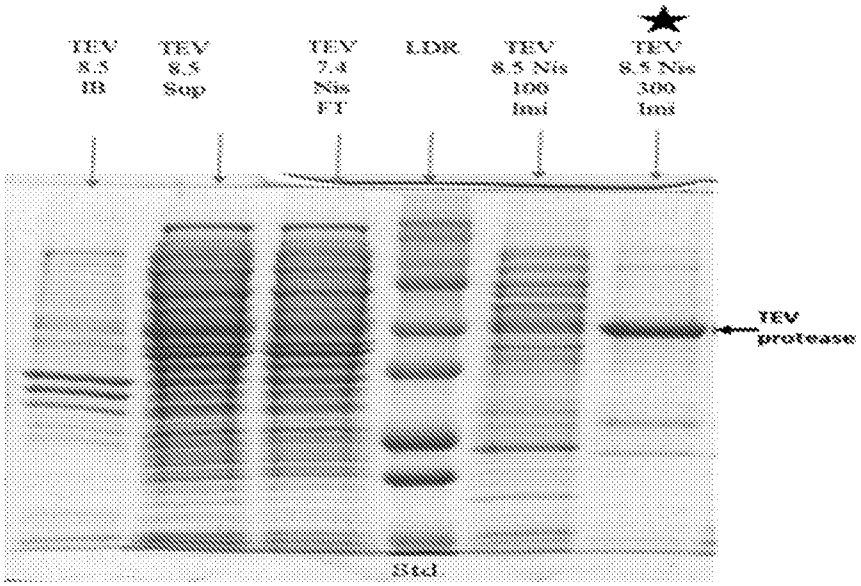

Figure-20a

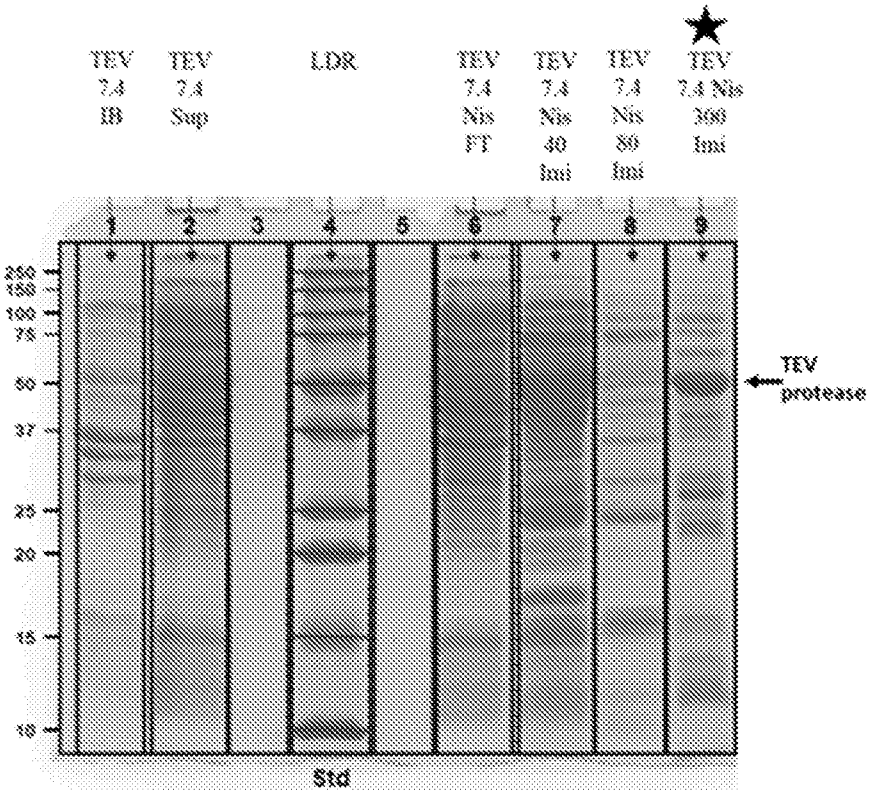

Figure-20b

Purification of 6×His-MBP tagged proteins by Ni-Sepharose Resin

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Na-Phosphate buffer+100mMNaCl+20mM Imidazol, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and upto 5 Cycles, Centrifuge and collect supernatant, 0.45 µm filtration

↓

Ni-Sepharose 6FF Column

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with lysis buffer and 0.45 µm filtered Post lysis Post lysis supernatant loaded on the column

↓

Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl+20mM Imidazol, pH 7.4); 5-6 CV

↓

Wash 2 with Buffer: (25mM Na-Phosphate buffer+300mM NaCl+20mM Imidazol, pH 7.4); 5-6 CV

↓

Wash 3 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV

↓

Wash 4 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV

↓

Elution with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)

↓

After elution purified 6×His-MBP tagged proteins concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 10 kDa/30kDa cassette and stored at 2-8 °C until further use

Figure-21

Purification of tagged proteins expressed in inclusion bodies

Cell Lysis

Harvested cell pellet 100 gram dissolved in ~2-3 ltr lysis buffer (25 mM Na-Phosphate buffer+100mMNaCl, pH 7.4) and Cell Lysed with Panda Homogenizer at 1000-1200 Bar for minimum 3 cycles and up to 5 Cycles, Centrifuge and collect supernatant and Post lysis pellet

Ammonium sulphate precipitation

Slowly add appropriate amount of Ammonium sulphate to make final concentration to 30% in supernatant. Allow it to stand for 20-30 min for complete precipitation. Centrifuge and collect precipitate

Protein Unfolding in Urea

Slowly dissolve ammonium sulphate precipitate/ IB's in to appropriate volume of 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 8M Urea until it dissolves completely. Filter with 0.22 µM filter and load on the column

↓

Ni-Sepharose 6FF Column (on column refolding and purification)

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 8M Urea and sample loaded on column

↓

Refolding- wash the column with reducing concentration of 25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ reducing concentration of Urea i.e 8M, 6M, 4M, 2M, 1M (each 5-6 CV)

↓

Wash 1 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4); 5-6 CV

↓

Wash 2 with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 40mM Imidazol); 5-6 CV

↓

Wash 3 with Buffer:(25mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 80mM Imidazol); 5-6 CV

↓

Elution with Buffer: (25mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)

↓

After elution purified 6×His-MBP tagged proteins concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 10 kDa/30kDa cassette and stored at 2-8 °C until further use

Figure-22

His-MBP tag from purified protein was removed using TEV protease enzyme as follows-

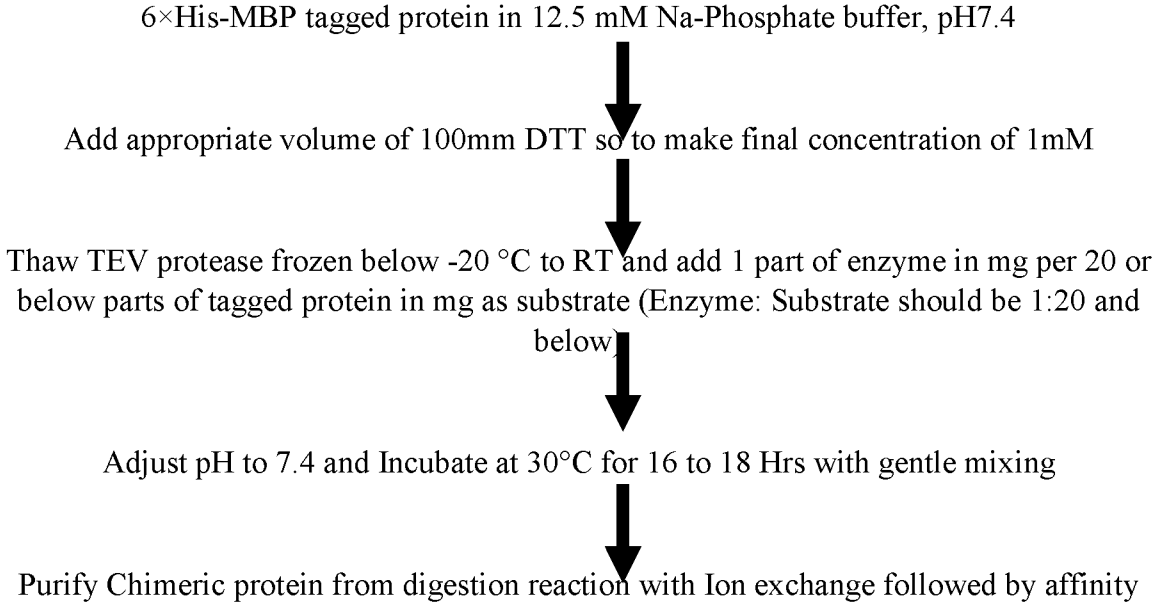

Figure-23

Part 1- Ion exchange (Strong anion exchange Capto Q) chromatography

Equilibration and Sample Loading: Charged Capto Q column equilibrated with 12.5 mm Na-Phosphate buffer pH 7.4 and digestion reaction was loaded on the column. Collect Flow through

↓

Elution 1 with Buffer: (12.5mM Na-Phosphate buffer+ 30mM NaCl, pH 7.4); 5-6 CV

↓

Elution 2 with Buffer: (12.5mM Na-Phosphate buffer+ 60mM NaCl, pH 7.4); 5-6 CV

↓

Elution 3 with Buffer: (12.5mM Na-Phosphate buffer+ 90mM NaCl, pH 7.4); 5-6 CV

FHBP V1.1 : PorA 307-311/exP1.4 and FHBP V1.1 : PorA 307-311/exP1.9 are present in flow through fraction. Whereas FHBP V3.45 M5 : PorA316-320/exP1.14, FHBP V2.19 M6 : PorA 316-320/exP1.4 and FHBP V1.14 : PorA307-311/exP1.9 are eluted in elution 2.

Fractions of interest are concentrated and diafiltered with 12.5mm Na-Phosphate buffer + 100mM NaCl + 5mM Imidazole using 5 kDa cassette and stored at 2-8 °C until further use

Figure-24

Part 2- Affinity chromatography Ni-Sepharose 6FF Column

Equilibration and Sample Loading: Ni-Sepharose column equilibrated with 12.5mm Na-Phosphate buffer + 100mM NaCl + 5mM Imidazole and purified chimera from Ion exchange chromatography loaded on the column to further purify Chimeric proteins

↓

Elution 1: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 30mM Imidazol); 5-6 CV

↓

Elution 2: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4 + 60mM Imidazol); 5-6 CV

↓

Elution 3: (12.5 mM Na-Phosphate buffer+100mM NaCl, pH 7.4+ 300 mM Imidazol)

↓

All the Chimeric proteins are eluted in Elution 1.

Elution 1/ Elution 2 containing final Drug substance i.e. Chimeric protein pooled if needed and concentrated and diafiltration carried out with 12.5 mM Na-Phosphate buffer pH7.4 using 5 kDa cassette.

Drug substance i.e. Chimeric protein sterile filtered and stored at 2-8 °C until further use/formulation

Figure-25

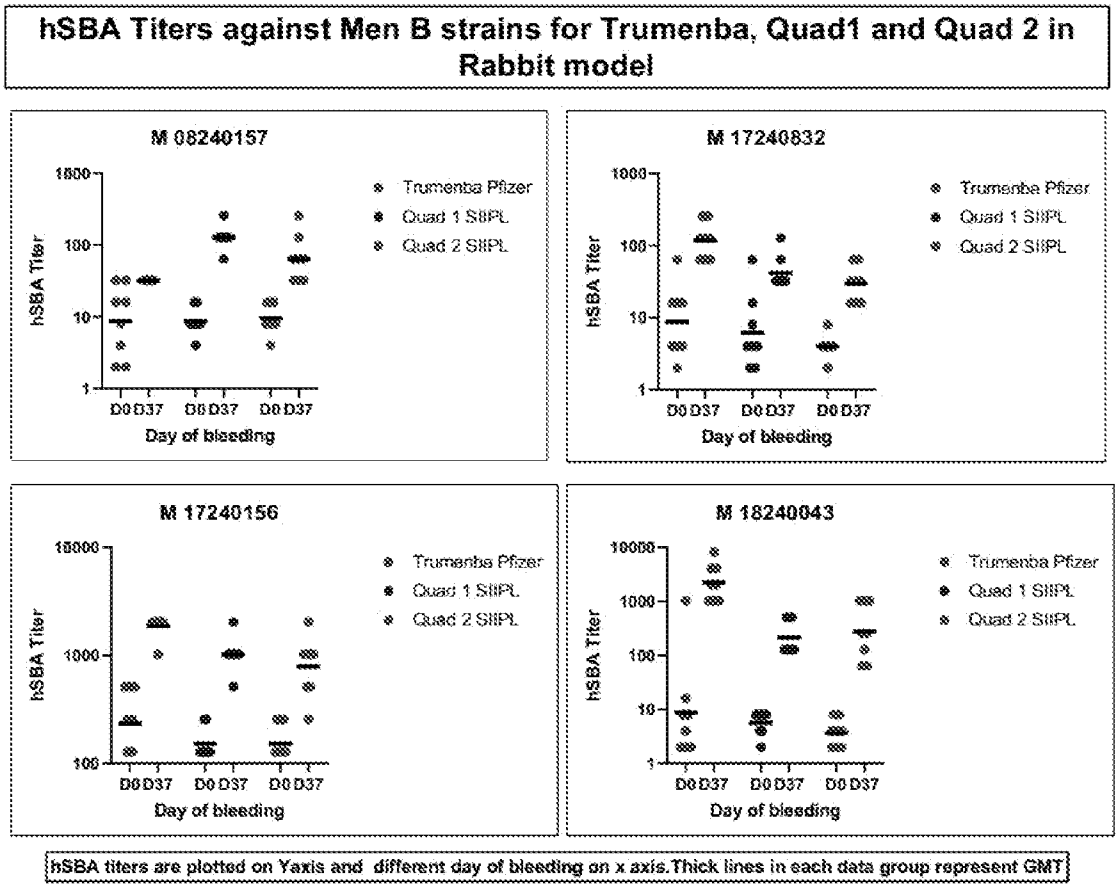
hSBA Titers against Men B strains for Trumenba, Quad1 and Quad 2 in Rabbit model
M 08240157
M 17240832
M 17240156
M 18240043
hSBA Titer
Day of bleeding
D0 D37
Trumenba Pfizer
Quad 1 SIIPL
Quad 2 SIIPL
hSBA titers are plotted on Yaxis and different day of bleeding on x axis.Thick lines in each data group represent GMT

Figure-26

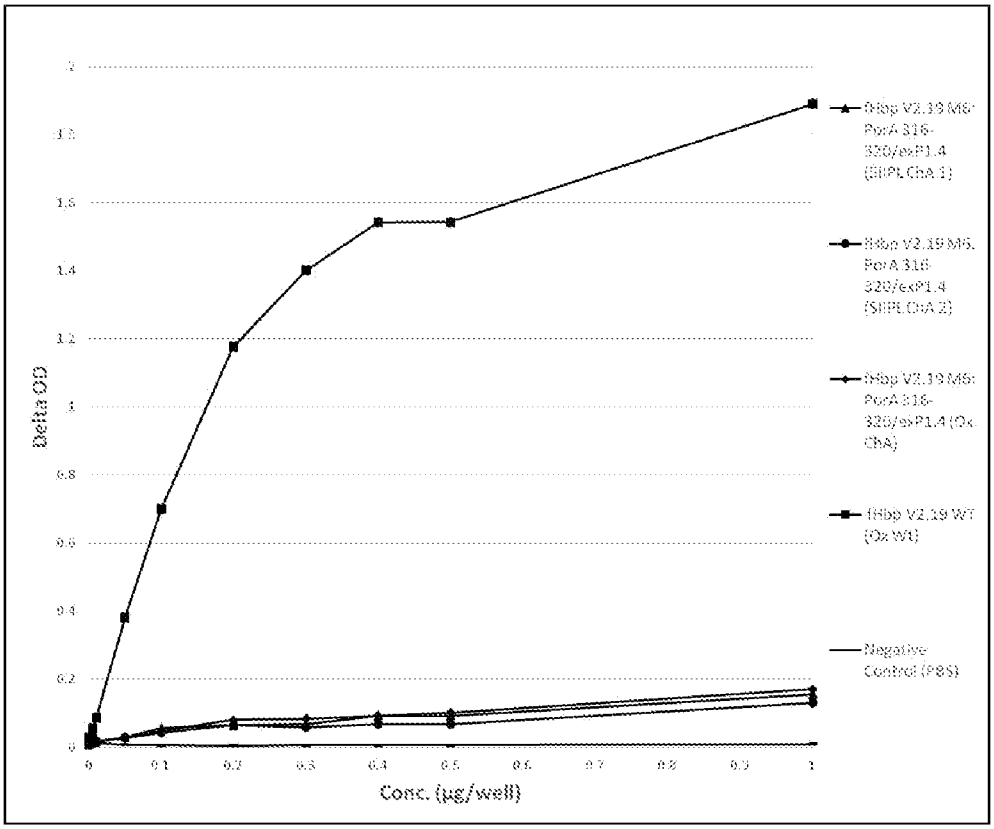

Figure-27

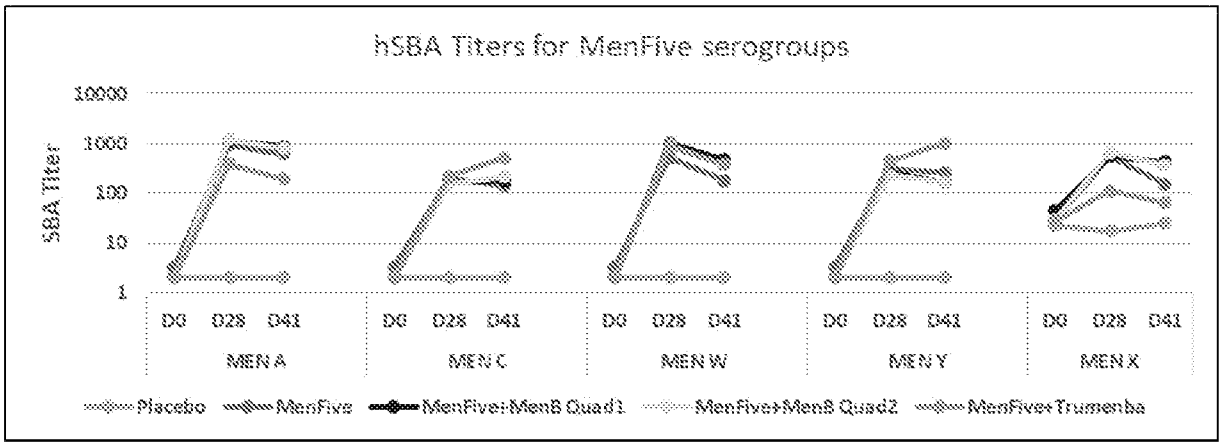

Figure-28

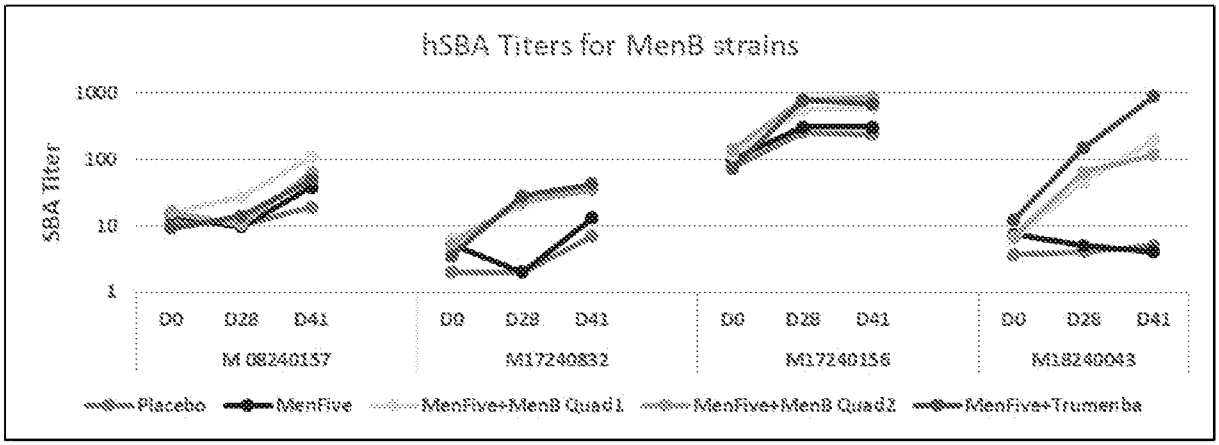

Figure-29

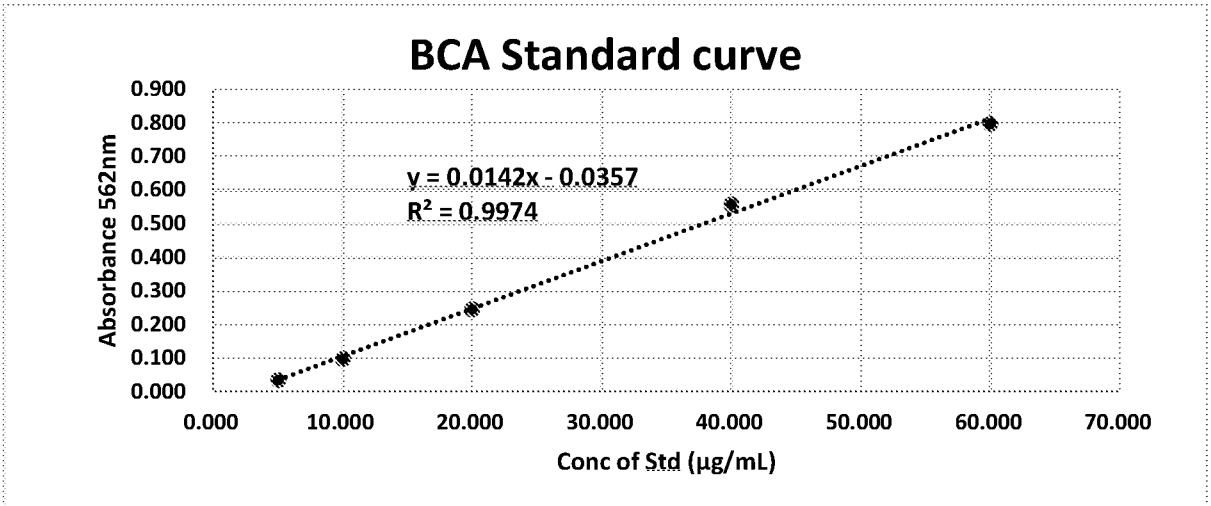

Figure-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050300

A. CLASSIFICATION OF SUBJECT MATTER
A61K39/095,A61P31/04,C07K14/22,C12N15/31,C12N15/63,C12N15/70
Version=2024.01
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, A61P, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

PatSeer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO2018042178A1 [OXFORD UNIVERSITY INNOVATION LIMITED] 08-03-2018 (08 MARCH 2018) refer to the whole document	1-16
Y	refer to the whole document	17-75
Y	CN110804102A [SUZHOU WEICHAO BIOTECHNOLOGY COMPANY LIMITED] 18-02-2020 (18 FEBRUARY 2020) refer abstract, claim 1	1-75
Y	WO2011024072A2 [NOVARTIS AG] 03-03-2011 (03 MARCH 2011) refer abstract, pages 9-12	1-75
Y	US20140323556A1 [THE CHILDRENS HOSPITAL OF PHILADELPHIA] 30-10-2014 (30 OCTOBER 2014) refer to the whole document	54-75

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18-07-2024	18-07-2024
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Akash Kumar Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050300

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed.

   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050300

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 76
because they relate to subject matter not required to be searched by this Authority, namely:

'see supplemental sheet'

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/IN2024/050300

Continuation of Claims found unsearchable(Box II)

The subject matter of claim 76 relates to a method for treatment of the human or animal body (method for inducing an immune response against Neisseria meningitidis serogroup B strain in an individual by administering to the individual a vaccine formula) which does not require an international search by the International Searching Authority in accordance with PCT Article 17(2)(a)(i) and [Rule 39.1(iv)].

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2024/050300

Citation	Pub.Date	Family	Pub.Date
WO 2018042178 A1	08-03-2018	US 20220112249 A1	14-04-2022
		EP 3506934 A1	10-07-2019
		JP 2019526261 A	19-09-2019
		CN 109890412 A	14-06-2019
		GB 2568440 B	17-08-2022
		AU 2017319218 A1	18-04-2019
		BR 112019003957 A2	25-06-2019
WO 2011024072 A2	03-03-2011	CN 104650241 A	27-05-2015
		JP 2015110653 A	18-06-2015
		ES 2562259 T3	03-03-2016
US 20140323556 A1	30-10-2014	EP 2970920 A1	20-01-2016
		WO 2014145578 A1	18-09-2014
		CN 105378074 A	02-03-2016
		AU 2014232879 A1	24-09-2015
		JP 2016512700 A	09-05-2016
		CA 2904366 A1	18-09-2014