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Nanomedicine for drug delivery 6 months traineeship report

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**Mannose functionalized Silk Fibroin Nanoparticles as a platform for the development of
innovative nanovaccines against tuberculosis**

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Abbreviations: ACN, Acetonitrile; AUC, Area Under the Curve; BCG, Bacillus Calmette-Guérin; CD, Cluster of Differentiation; CF-NHS, 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester; CRD, Carbohydrate Recognition Domain; CTLR, C-type Lectin Receptors; DC-SIGN, Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin; DLS, Dynamic Light Scattering; HCl, Hydrochloric Acid; HPLC, High-Performance Liquid Chromatography; IFN- γ , Interferon γ ; IGRAs, Interferon-Gamma Release Assays; IME, Imine Methoxy Ethyl; LiBr, Lithium Bromide; L-FDVA, Na-(2,4-Dinitro-5-fluorophenyl)-L-valinamide; M1, Monosaccharide Mannose; M4, Tetrasaccharide Mannose; Man LAM, Mannose-Capped Lipoarabinomannan; MHC, Major Histocompatibility Complex; MR, Mannose Receptors; MTB, Mycobacterium tuberculosis; MQ water, Milli Q Water; NTA, Nanoparticles Tracking Analysis; OPA, O-Phthalaldehyde; PSA, Phenol Sulfuric Acid; RPLC, Reverse Phase Liquid Chromatography; SFNs, Silk Fibroin Nanoparticles; TACO, Tryptophan Aspartate-Containing Coat Protein; TB, Tuberculosis; TFA, Trifluoroacetic Acid.

1.Introduction

1.1.Tuberculosis: Disease Overview and Biological Background

World Health Organisation (WHO) classifies TB as the world's leading cause of death from a single infectious agent. TB is caused by *Mycobacterium tuberculosis* MTB affecting mainly the lungs . The major route of the transmission is through inhaling the aerosolized bacilli from the air. About 5% of infected individuals are at risk of developing the disease in the first two years, after which the risk is lower. The number of incident cases increased to 10.8 million in 2023 (as shown in Figure 1) from 10.1 million in 2021. Although, TB can affect any person regardless of gender or age, it was found that the highest burden of the disease affects adult male older than 15 years old, as shown in Figure 2

The treatment of the TB includes 1-6 month regimen with a cure rate about 85%. However, if not treated the case fatality rate is about 50 % (1).

Estimated TB incidence rates, 2023

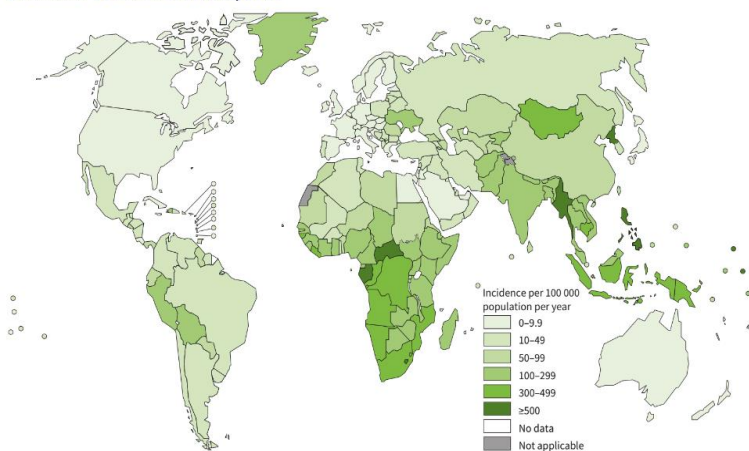


Fig.1. Estimated TB incidence rates
Link: [Global Tuberculosis Report 2024](#)

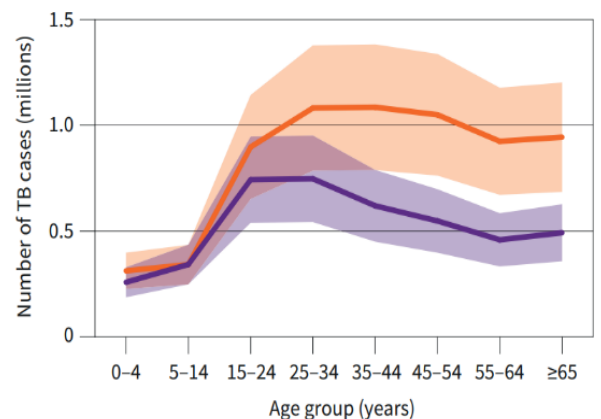


Fig.2. Estimated TB incidence according to gender and age groups
(Male in **orange**, Female in **purple**)
Link: [Global Tuberculosis Report 2024](#)

MTB is classified as an aerobic intracellular microorganism that grows in highly oxygenated tissues, mainly the lungs. MTB is an acid-fast stained microorganism since its thick, lipid-rich cell wall does not stain well with Gram stain (2). The cell envelope of MTB has a complex array of glycolipids, such as mannose-capped lipoarabinomannan (ManLAM) which has significant roles in the pathogenesis of the disease through mediating phagocytosis and phagosome maturation arrest (3). There are several diagnostic tests for MTB such as bacterial culture from sputum, nucleic acid amplification tests (NAAT), and immunological tests such as interferon-gamma release assays (IGRAs). However, each diagnostic method has its own limitations. For example, bacterial culture is time-consuming. Furthermore, IGRAs cannot distinguish between active and latent infection since the immunological response is similar in both cases. Finally NAATs cannot differentiate between live and dead bacilli specimen (4)(5).

MTB life cycle starts by entering the airways through nose or mouth where in most case reach the lung alveoli. ManLAM is recognized by pattern recognition receptors on immune cells residing in the alveoli, such as mannose receptors (MRs) and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), leading to the phagocytosis of MTB. Normally, macrophages complete their

maturation cycle and fuse with lysosomes lead to the eradication of the micro-organism. However, in many cases, MTB interferes with the maturation of macrophages and the subsequent acidification via ManLAM and TACO protein (Tryptophane aspartate-containing coat protein) (6). As a result, infected immune cells, such as macrophages, cannot eliminate the bacilli and eventually become reservoirs for MTB. Infected monocytes and macrophages release various cytokines and chemokines, recruit additional monocytes and granulocytes to the site of infection. Although this cells influx can enhance the MTB transfer between immune cells, it also helps initiate the adaptive immune response through migration of infected dendritic cells to lymphoid organs, where they prime naïve T cells which are the main immune mediators against MTB and lead to the initiation of the adaptive immune system.

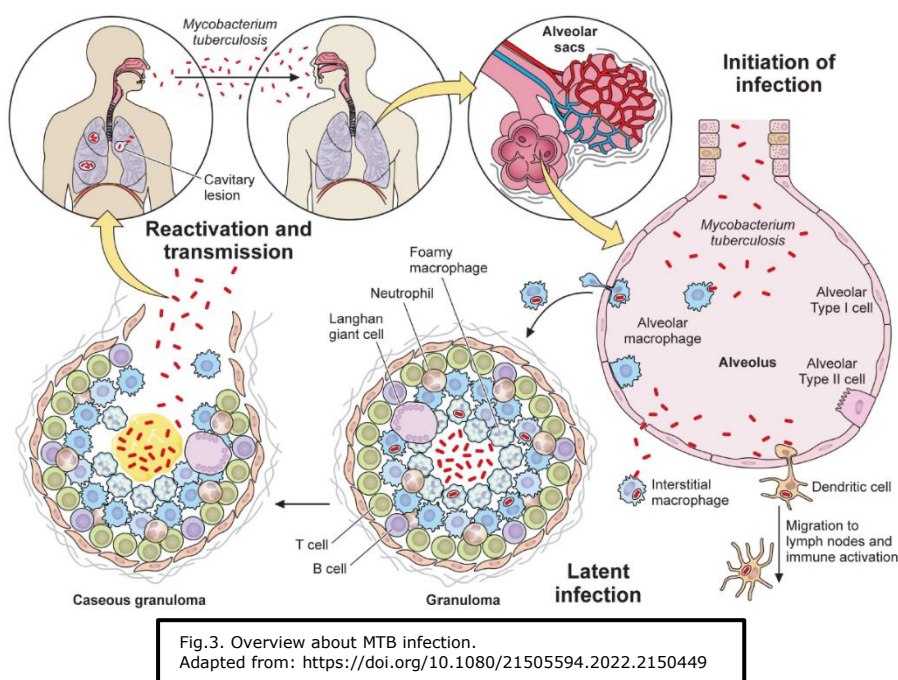


Fig.3. Overview about MTB infection.
Adapted from: <https://doi.org/10.1080/21505594.2022.2150449>

Activation of the adaptive immune response requires transport of live bacteria to the draining lymph node. Some studies have proposed that MTB can interfere with antigen presentation on MHC, leading to impairment or delay of the adaptive immune response (7). The adaptive immune response is carried by two main T cells subtypes $CD4^+$ T and $CD8^+$ T cells. $CD4^+$ T cells secrete $IFN-\gamma$, which activates the infected macrophages in order to restrict intracellular bacterial replication. While $CD8^+$ T cells contribute through their cytotoxic effect on the MTB infected cells by granule exocytosis pathway (8). Infected and uninfected macrophages, alongside neutrophils, dendritic cells, B cells, and T lymphocytes, aggregate together with the aim of inhibiting replication and isolating MTB, leading to the formation of a solid granuloma, which is a main hallmark of TB infection. While, solid granulomas limit the spread of the infection and limit bacterial growth, they can also prevent the immune system from completely eradication of the bacteria, resulting in a latent infection (9). A brief overview of MTB infection is illustrated at Figure 3. Thus the main goal of vaccination is to accelerate the activation and recruitment of the adaptive immune system in order to prevent the persistence of the bacteria (10).

1.2. Current Strategies in tuberculosis vaccination

Vaccines against MTB can be classified according to the time and purpose of administration into: prophylactic vaccines and therapeutic vaccines. Prophylactic vaccines (Pre-exposure) are intended for preventing infection and are subclassified into Priming and boosting doses, as shown in Figure 4. Priming doses, such as BCG, aim at educating naïve t-cells for the first time, while boosting doses are additional doses administered after the priming dose to stimulate the memory cells. Boosting doses can be of the same type as priming vaccine (Homologous) or of a different type (Heterologous) (11). In the case of MTB, using heterologous vaccination approach appears to be more promising. On the other hand, therapeutic vaccines, still under investigation, are used either to clear a current infection, stop its progression or preventing recurrence of the infection (8)

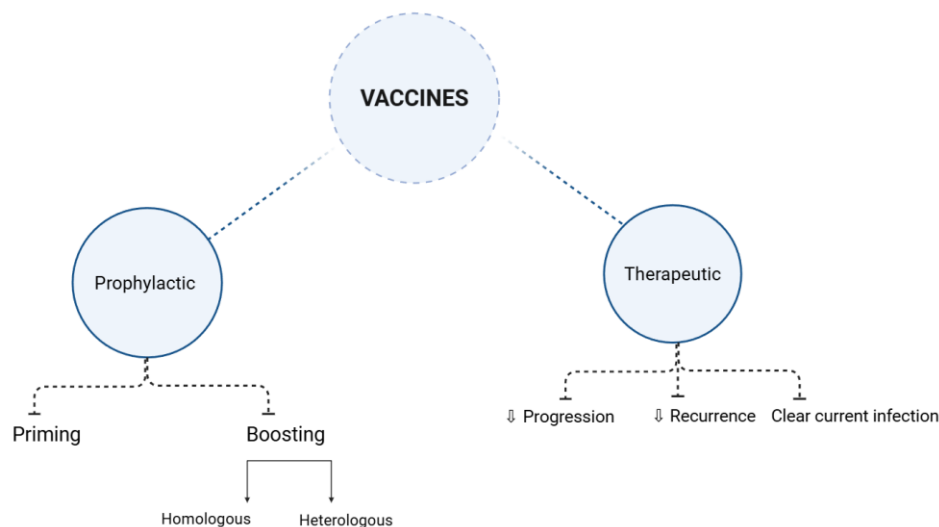


Fig.4. Classification of Vaccines against TB according to time and purpose of use

BCG ,the only approved vaccine against TB, is a live attenuated vaccine from strain of *Mycobacterium bovis* developed in the Pasteur institute in Paris by scientists Albert Calmette and Camille Guérin(11). BCG is proven to induce pronounced immunological protection. This protection is due to the production of CD4⁺ T memory cells which rapidly secrete Interferon γ and other Th1 cytokine leading to protection against the subsequent infection. Additionally, it has been recently reported that BCG vaccine also enhances the ability of the innate immune system to produce cytokine and clear MTB and this effect is called 'Trained immunity'. Surprisingly, these innate immune cells exert enhanced reaction towards unrelated pathogens as well (12).

However, the induced immunological protection by BCG is reported to be effective mainly in childhood and gradually wanes over time leading to variable degrees of protection for adults (13). This is considered the main reason for the transmission of infection. Furthermore, Several articles have reported that revaccination with booster shoots of BCG induced no or a limited efficacy (14,15). Another problem is that Immunocompromised infants, such as those with HIV, have no / little benefit from BCG. At the same time it can give rise to disease caused by the vaccine itself called BCGosis (16). Additionally, HIV patients are 20 - 30 fold more risk to get TB if they are infected with MTB (17).

For the aforementioned reasons and given the slow decline of the TB infections, there is a pressing need for a vaccination (Prophylactic and therapeutic) that can provide protection to different age groups alongside with immunocompromised individuals.

To address this need, three main types of tuberculosis (TB) vaccines are currently under clinical development: whole-cell vaccines, viral-vectored vaccines, and subunit vaccines. Whole-cell vaccines are derived from the entire *Mycobacterium tuberculosis* (Mtb) organism, which may be either inactivated (killed) or attenuated (weakened). These vaccines are considered polyantigenic, as they present multiple antigens to the immune system, potentially eliciting a broad and effective immune response. Viral-vectored vaccines, on the other hand, utilize non-replicating viruses—often genetically modified—to deliver specific TB antigens into host cells (10,18). These vaccines typically do not require adjuvants, as the viral vector itself is sufficient to activate the immune response. While, subunit vaccines consist of antigenic proteins or even fragments of the proteins needed to induce an efficient immunological response (8,19). When compared to live attenuated vaccines, subunit vaccines have a better safety profile as they lack non-essential elements, reducing the risk of causing active infections or undesired immune response. Subunit vaccines can be considered safer for the immunocompromised patients such as: HIV patient vaccines. However, since they are formed only of just a fraction of the antigens found on the whole organism, they usually have poor immunogenicity and require multiple doses of administration. This problem is usually solved through using adjuvants, substances that can increase the body's immune response to the antigens. Adjuvants are classified according to their function into two main types:

- 1) Immune potentiators such as ligand that can activate the pattern recognition receptors on antigen presenting cells (APC's) such as, C-type lectin receptors (CTLRs) ligands and Toll- like receptor.
- 2) Delivery systems with the aim of concentrating the display of the antigenic protein as well as colocalizing the antigenic protein with the immune potentiator if present. (8,20).

1.3. Targeted Nanoparticle Platforms for TB Vaccination: Mannosylated Silk Fibroin Nanoparticles (SFNs) .

Mannosylated NPs present a promising strategy for the preparation of subunit vaccines, because this system combines both types of adjuvants. On one hand mannosylation can allow for a better uptake by APC's such as: macrophages and dendritic cells.

APC's highly express two types of pattern recognition receptors: MRs and DC-Sign receptors. MRs are endocytic CTLR composed of type I transmembrane proteins, with their C-terminus located in the cytoplasm. MRs contain eight carbohydrate recognition domains (CRDs), though carbohydrate binding primarily occurs through CRDs 4 to 8, with CRD 4 exhibiting the highest affinity for monosaccharides. Among various monosaccharides, MRs and DC-SIGN display the highest affinity for D-mannose and L-fucose. (21). However, DC-SIGN receptors belong to another class of CTLRs and function as type II transmembrane proteins, with their N-terminus located intracellularly and their C-terminus exposed extracellularly (22). DC-SIGN is primarily expressed on dendritic cells and plays a crucial role in antigen recognition and pathogen binding (23).

Several factors influence mannose binding affinity to APC's receptors such as valency, density and the spatial arrangement of mannose units. Regarding valency, increasing the number mannose units capable of simultaneously interacting with receptors can improve the uptake. Mannose density on NPs also affects receptor affinity; in monovalent systems (when the NPs are functionalized using monosaccharide) , higher density leads to increased receptor binding and uptake, whereas in multivalent systems, this effect is less pronounced. The spatial arrangement of mannose ligands further influences receptor

binding. For instance, a parallel tri-mannose ligand exhibits higher affinity for MRs, whereas a tri-antenna mannose ligand favors DC-SIGN receptors. Notably, higher-order mannose ligands (tetra- and pentavalent) do not necessarily exhibit stronger binding to MRs than simpler structures (21,24).

On the other hand, SFNs allow for concentration of the antigen on the surface of the nanoparticles mimicking the normal size of the Pathogen. They also enable co-localization of CTLR ligands (mannose moieties) with the antigenic protein.

Moreover, SFNs, exhibit several other advantages such as biodegradability, biocompatibility, and versatile processability in aqueous environments as well as easiness surface functionalization(25). Furthermore, silk fibroin is approved by the FDA as a biodegradable polymer and also classified as Generally regarded As Safe material for medical devices and wound healing(26).

For this formulation, we evaluated the potential advantages and disadvantages of PEGylation. On one hand, PEGylated SFNs would likely exhibit improved colloidal stability due to steric hindrance and reduced recognition by immune cells, thereby enabling prolonged circulation in the bloodstream, an advantage for intravenous (IV) administration(27).

However, in our case, the SFNs are designed for intradermal injections since skin is normally rich in immune cells, especially APCs such as dendritic cells and Langerhans cells as well as allow to targeting proximity to lymph nodes and promoting T cell activation (similar to the BCG administration route)(28).

The primary purpose of these functionalized SFNs is to enhance uptake by APCs via targeting MR and DC-SIGN, and to improve the immunogenicity of the peptide through a controlled immune response induced by the SFNs themselves (21). Additionally, it has been reported that non-PEGylated SFNs can induce a more robust immune response compared to PEGylated ones (29). SFNs also possess inherent colloidal stability, attributed to the β -sheet structure of the silk fibroin protein (30).

For all the above reasons—and despite the generally lower stability of non PEGylated SFNs, we opted not to PEGylate the SFNs, given the intended route of administration and the specific objectives of this formulation. According to the literature, several chemical techniques have been developed in order to functionalize nanoparticles with sugar derivatives, such as click chemistry, reductive amination, imino-methoxyethyl (IME) glycosylation, EDC-NHS, NHS bifunctional linkers, and isothiocyanate–amine coupling (31–34). The choice of conjugation strategy depends on several factors, for example: the availability of functional groups on the NP surface, the stage of functionalization (pre- or post- NP synthesis), as some reaction conditions can affect NP characteristics and stability, and the overall reaction yield. In this study, click chemistry via the azide/alkyne approach has been adopted, since this reaction is considered a highly selective and orthogonal technique. Additionally, IME, an indiscriminate glycosylation technique, which is a milder conjugation method, has been employed to allow direct glycosylation of primary amine groups on lysine side chains (35).

After optimizing the mannosylated SFNs to determine which targeting moiety better enhances binding and/or uptake by APCs, short peptides—approximately 10 amino acids —will be attached to the surface of the SFNs using a click chemistry approach. These peptides will be derived from highly antigenic proteins such as Ag85(Most antigenic peptide in MTB) (36), which plays a key role in maintaining the stability of the mycobacterial cell wall, and ESAT-6 (an antigenic protein that is not present in BCG) (37).

2. Materials and Methods:

2.1. Chemicals and Reagents:

5(6)-Carboxyfluorescein NHS Ester, Acetonitrile, Azide-Fluor 545, Copper Sulfate, Dimethylformamide, L-Lysine Hydrate, Na-(2,4-Dinitro-5-Fluorophenyl)-L-Valinamide (L-FDVA), Phenol, Sodium Ascorbate, Sodium Tetraborate Decahydrate, Thyroglobulin (from Bovine Thyroid), Trifluoroacetic Acid, Trichloroacetonitrile, and 4-Dimethylaminopyridine were all purchased from Sigma-Aldrich (Germany). O-Phthalaldehyde was obtained from TCI (Shanghai, China). Mercaptopropionic Acid was purchased from Thermo Scientific (USA). Acetic Anhydride, Sodium Bicarbonate, and Triethylamine were procured from Carlo Erba (Italy). 1,5,7-Triazabicyclo[4.4.0]dec-5-ene, Lithium Bromide, and Sulfuric Acid were purchased from Fluka (Italy).

2.2. Organic chemistry:

The Synthesis of M1 and M4 moieties were prepared following our lab's standard protocol, with the key chemical reactions summarised below:

- As shown in figure 5, in the 1st approach (IME approach), mannose derivatives (M1 and M4) bearing a thiocyanomethyl group at the anomeric position were synthesized for glycosylation via the IME method. Briefly, after chlorination of peracetylated D-mannose, the subsequent treatment with thiourea replaced the chlorine atom with a thiopseudourea moiety. The final conversion into the desired thiocyanomethyl group (M1--SCH₂CN) was achieved using sodium bisulfite, potassium carbonate, and chloroacetonitrile (overall yield: 41%). After the chemoenzymatic synthesis of the 6-deprotected SCH₂CN derivative, it was condensed with the trisaccharide derivative, previously activated as a trichloroacetimidate, to obtain the M4-SCH₂CN compound

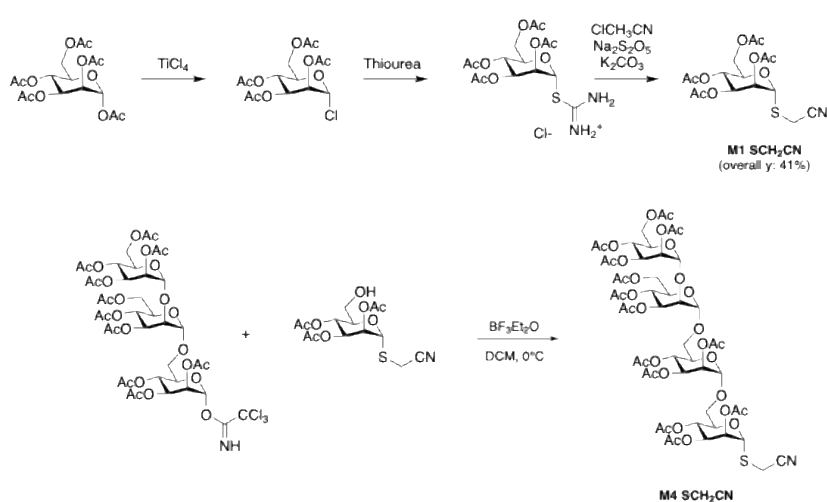


Fig.5. Chemical scheme for synthesis of M1 and M4 moieties for IME chemistry

- As shown in figure 6, in the second approach (click chemistry), M1 and M4 derivatives bearing an azide group at the anomeric position were synthesized for subsequent click reaction. In this case, fully acetylated mannose was directly condensed with 3-chloropropanol under heating conditions. The resulting intermediate was then treated with sodium azide in DMF at 70 °C for 4 hours to yield the M1-azide derivative (yield: 85%). Similarly to the first approach, after chemoenzymatic

synthesis of the 6-deprotected azide derivative, it was condensed with the trisaccharide derivative, previously activated as a trichloroacetimidate, to obtain the M4-azide compound

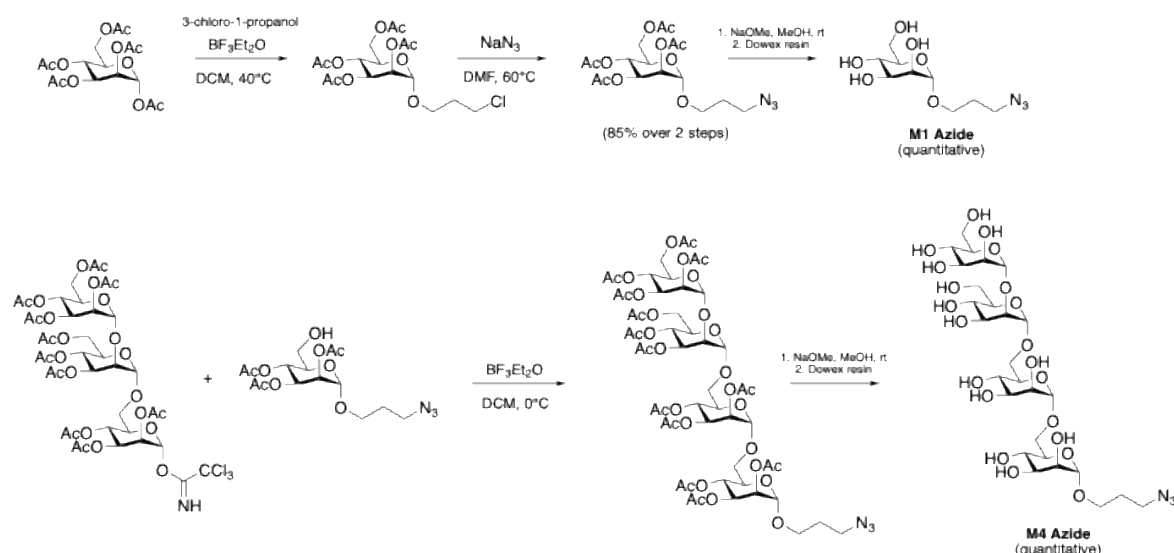


Fig.6. Chemical scheme for synthesis of M1 and M4 moieties for click chemistry

2.3. SILK fibroin NP preparation

The SFNs were prepared by Dr. Elia BARI, a collaborator from the university of Eastern Piedmont (38). Briefly, cuts of *bombyx mori* cocoons with dimensions 1x1 cm were boiled at 100 °C in an aqueous solution of Na₂HCO₃ (0.02M) for half an hour to separate the fibroin fibers from sericin. The separated bulk fibroin fibers were then washed by deionized water and dried at ambient temperature. The dried fibroin was then solubilized using a concentrated chaotropic solution of Lithium Bromide (LiBr, 9.3 M for 4 hours), subsequently dialyzed for 3 days against MQ water to remove LiBr. The dialyzed sample was then diluted to 1.5% w/v concentration. Finally, the SFNs were prepared by dropwise addition of the diluted solution of fibroin (1.5% w/v) into acetone with at ration of fibroin:acetone of 1:5.

2.4. Functionalization of SFNs :

2.4.a. Quantification of lysine content in blank SFNs

To accurately adjust reactant amounts for glycosylation, it was necessary to estimate the lysine content in SFNs. After that, the glycosylation of SFNs via the two chemical approaches will take place according to the scheme expressed in figure 7.

Firstly, a defined mass of SFNs was fully hydrolyzed to release free amino acids, including lysine. A derivatization step was applied to enhance amino acid detection. A calibration curve of derivatized lysine was then generated to identify and quantify the lysine peak in the hydrolysate. The hydrolysis protocol used was adapted from methods reported by Langrock et al. and Lioi et al., ensuring complete amino acid release from the protein nanoparticles.(39,40).

Gas-phase hydrolysis was used to determine lysine content. SFNs were dispersed in MQ water at 1 mg/mL, briefly sonicated (30 s), and 150 µL of the dispersion was transferred into a glass insert and dried under nitrogen. The dried insert was then placed inside an HPLC vial containing 300 µL of 6 M HCl (amino acid analysis grade), sealed with an acid-resistant cap, and incubated at 110 °C for 24 h. After hydrolysis, the sample was resuspended in 15 µL of MQ water, briefly sonicated, and the insert was inverted inside a centrifuge tube and spun for 15 s to collect the hydrolysate.

For derivatization, 5 μL of the hydrolysate was mixed with 16 μL of MQ water, 8 μL of 1 M Na_2HCO_3 , and 32 μL of 36.7 mM L-FDVA (Na-(2,4-dinitro-5-fluorophenyl)-L-valinamide). The reaction was carried out at 40 $^\circ\text{C}$ with agitation for 1.5 h. It was then quenched with 8 μL of 1 M HCl and diluted with 200 μL of acetonitrile and 731 μL of MQ water to a final volume of 1 mL.

A calibration curve for lysine was prepared by making serial dilutions (2, 5, 10, 20, 50, 100 μM) from a 1 mM stock solution. Briefly, 10 μL of a 100 mM lysine stock solution was mixed with 40 μL of MQ water, 20 μL of 1 M Na_2HCO_3 , and 165 μL of L-FDVA (36.7 mM in acetone) in an Eppendorf tube. The reaction was incubated at 40 $^\circ\text{C}$ with agitation for 1.5 h, then stopped by adding 20 μL of 1 M HCl. Subsequently, 200 μL of MQ water and 545 μL of acetonitrile were added to reach a final volume of 1 mL.

For RPLC-UV analysis, mobile phases were: (A) water + 0.1% TFA and (B) acetonitrile + 0.1% TFA. A gradient elution was applied from 80:20 to 10:90 (A:B) over 30 minutes at a flow rate of 500 $\mu\text{L}/\text{min}$, followed by reconditioning at 80:20 for 30 minutes. The injection volume was 4 μL , and detection was performed at 340 nm. The column used was a Kinetex C18, 2.6 μm particle size, 100 $\text{\AA}$ pore size, 100 $\times$ 4.6 mm.

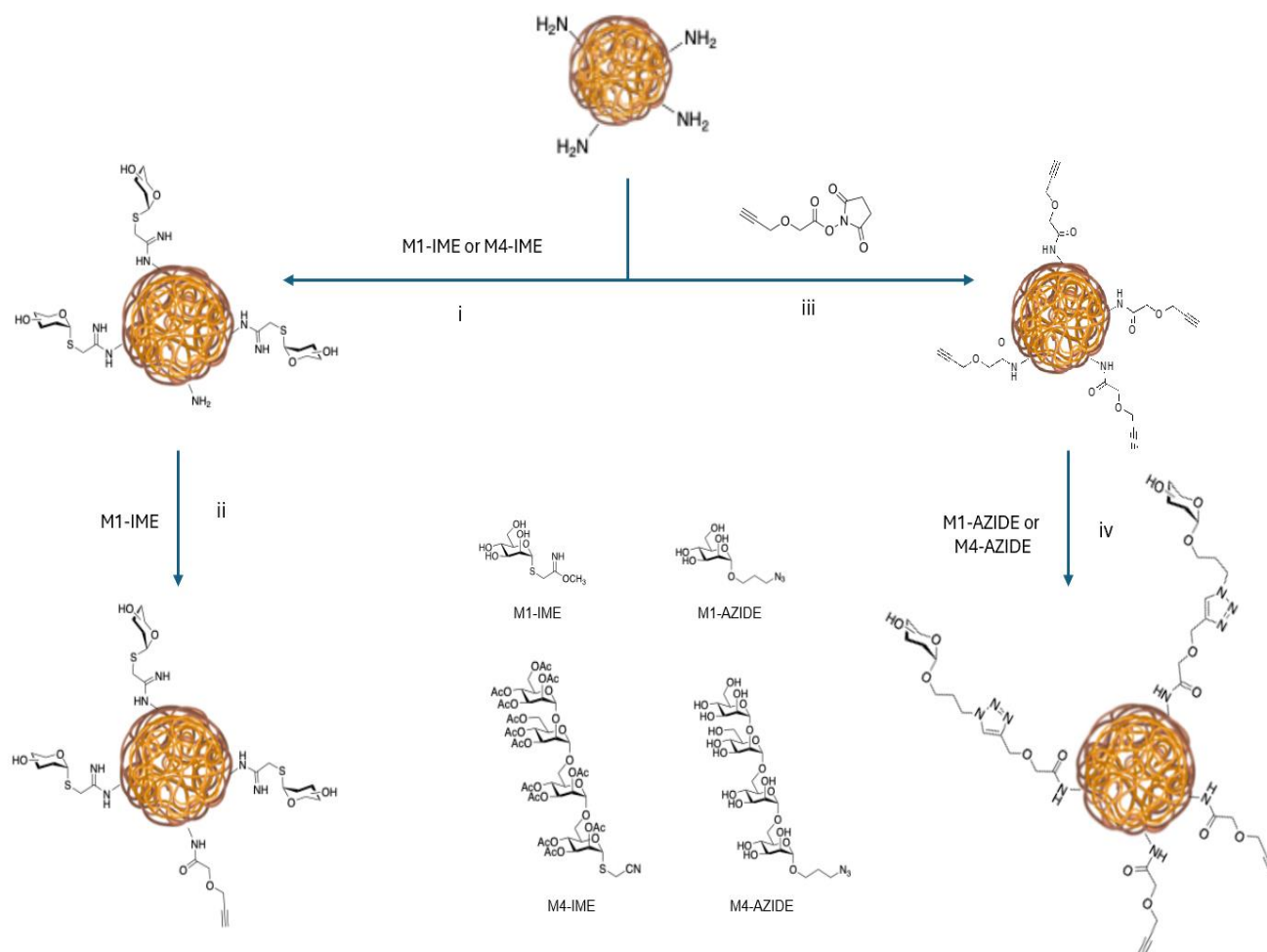


Figure 7 Chemical scheme for Glycosylation of SFNs

2.4.b. 1st Formulation (IME approach) – M1- and M4- has a thiocyanomethyl group

2.4.b.i. Glycosylation of SFNs with mono & tetrasaccharide mannose

Firstly, M1 & M4 with SCH₂CN were activated into M1 & M4 with reactive IME group via treatment with sodium methoxide in methanol as a one-step activation deprotection reaction. Secondly, in four different round bottom flasks, 25 mg of SFNs were homogeneously dispersed into 2.5 ml of borate buffer of a pH= 9.5 (100mM). The first three flasks were reacted with different amounts of mannose M1-IME (10,20,40 nmol per mg of NPs). The fourth one was instead reacted with the M4-IME (20 nmol per mg of NPs). The reaction were left under stirring for 24 hours followed by 3 cycles of successive centrifugation–resuspension with MQ water (each cycle is 14000 RPM for 15 minutes) followed by freeze-drying.

2.4.b.ii. Modification of the accessible lysine left into a triple bond linker

With the intended goal of achieving surface heterodecoration of the nanoparticles by targeting lysine residues for dual functionalization, both with mannose moieties and with an antigenic peptide, we explored the possibility of selectively modifying the remaining lysines using a distinct chemical strategy. Since antigenic peptides can be synthesized bearing an azide group, we investigated the introduction of an alkyne functionality onto the SFN surface to enable conjugation via click chemistry.

To this end, we selected SFNs decorated with a lower degree of glycosylation (reacted with 10–20 nmol/mg of M1 mannose) to preserve free amine groups available for further modification. A total of 15 mg of these partially glycosylated SFNs were homogeneously dispersed in 1.5 mL of phosphate buffer (100 mM, pH 7.4). To the dispersion, an NHS-activated linker bearing a terminal alkyne (40 nmol/mg) was added to selectively target the unreacted amines. The reaction was allowed to proceed under continuous stirring for 24 hours at room temperature, followed by washing and lyophilization as described in Section 2.4.b.i.

2.4.c. 2nd Formulation (Click chemistry Approach)- M1 and M4 has an azide group

2.4.c.i. Modification of accessible amine groups with a triple bond linker

The same NHS-activated alkyne linker introduced in the previous paragraph—originally considered for potential peptide conjugation—can also be employed to attach mannose moieties having an azide group, according to the second proposed strategy (click chemistry).

To this end, 30 mg of SFNs were homogeneously dispersed in 3 mL of phosphate buffer (100 mM, pH 7.4). The NHS-activated linker bearing a terminal alkyne was added in a quantity corresponding to 1 equivalent (40 nmol/mg), allowing it to react with the primary amine groups exposed on the nanoparticle surface through NHS esterification, forming stable amide bonds. The reaction mixture was stirred continuously at room temperature for 24 hours. After completion, the particles were washed and lyophilized as described in Section 2.4.b.i.

2.4.c.ii. Glycosylation of SFNs with M1 & M4- mannose

To evaluate the feasibility of conjugating azido-functionalized mannose derivatives to the SFNs via click chemistry, and in line with the broader objective of achieving surface heterofunctionalization, we

intentionally performed the reaction under sub-stoichiometric conditions, aiming to preserve a fraction of unreacted alkyne groups for potential further functionalization (e.g., peptide attachment).

Specifically, 5 mg of lyophilized SFNs functionalized with the alkyne linker were dispersed in 0.5 mL of phosphate buffer (100 mM, pH 7.4) in two separate Eppendorf tubes. The dispersions were sonicated for 2 minutes and then left to equilibrate under stirring for 30 minutes. To each tube, CuSO₄ (25 nmol/mg) and sodium ascorbate (120 nmol/mg) were added as the catalytic system for the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC).

Subsequently, M4-b azide (4 nmol/mg) was added to the first tube, while M1 azide (4 nmol/mg) was added to the second, allowing the reaction to proceed under continuous stirring. After completion, the nanoparticles were purified and lyophilized as described in Section 2.4.b.i

2.5. Characterization of Nanoparticles: Particle Size and Zeta-potential:

Particle size measurements were performed using Nanoparticle Tracking Analysis (NTA, NanoSight NS300, Malvern Panalytical). Prior to measurement, SFNs were dispersed in MQ water at a concentration of 0.1 mg/mL, sonicated, vortexed, and then filtered through 0.45 µm nylon filters (Whatman). Five replicates were recorded per sample to ensure reproducibility.

Zeta potential measurements were carried out using Dynamic Light Scattering (DLS, Litesizer, Anton Paar). SFNs were dispersed in 1 mM KCl solution to a final concentration of 1 mg/mL, sonicated, vortexed, left to equilibrate overnight, and then filtered using 0.45 µm nylon filters (Whatman). Each sample was measured in triplicate.

2.6. Quantification of Glycosylation

2.6.a. Direct quantification: Phenol Sulfuric Acid (PSA) method:

Briefly, a homogenous dispersion of Blank SFNs and mannose (M1) functionalized SFNs were dispersed at MQ water at a conc of 1 and 2 mg/ml. 1 ml of the homogenous dispersion is added to a test tube followed by the addition of 5 ml of sulfuric acid (97%) and immediately 1 ml of 5% phenol solution is added followed by gentle shaking then a yellow colored solution should be developed and the absorbance of the solution is measured by UV-Vis spectrophotometer at 490 nm.

A calibration curve from (2 to 150 nmol/ml) was constructed from the mannose (M1) with thiocyanomethyl linker that was used during the glycosylation reaction

2.6.b. Indirect quantification:

2.6.b.1. 1st Formulation (IME approach):

2.6.b.1.A/ O-phthalaldehyde (OPA) assay

The method used closely follows literature protocols with minor modifications (35). Fresh OPA solution was prepared immediately before measurements by dissolving 25 mg of OPA in 250 µL methanol, then diluting to 25 mL with borate buffer (pH 9.5), followed by the addition of 50 µL mercaptopropionic acid. The solution was protected from light. In a 96-well plate, 20 µL of SFNs (2 mg/mL) was mixed with 290 µL of OPA solution and shaken for 2 minutes to form fluorescent indole derivatives. Fluorescence was measured using a microplate reader at 460 nm emission with 355 nm excitation. A calibration curve was generated using thyroglobulin at varying concentrations (0.1, 0.5, 10, 20, 50, and 100 nmol/mL) to

quantify free amine groups. Thyroglobulin contains 75 primary amines per monomer (equivalent to 247.4 nmol per mg), serving as a reference standard. SFNs with varying degrees of glycosylation were compared: non-functionalized SFNs and functionalized SFNs reacted with different sugar concentrations: 10, 20, and 40 nmol/mg. Prior to analysis, samples were pre-dissolved in 9.3 M LiBr and incubated at 60 °C for 5 hours to enhance lysine accessibility for reaction with OPA.

2.6.b.1.B/ 5(6)-Carboxyfluorescein *N*-hydroxysuccinimide ester (CF-NHS) dye (Quantifying the accessible lysines on the surface of glycosylated SFNs)

A calibration curve for the free dye was generated using five concentrations (0.5, 1, 2, 5, and 10 nmol/mL). SFNs glycosylated at 10 and 20 nmol/mg were selected to maximize antigenic peptide loading. For each formulation, 5 mg of SFNs were dispersed in phosphate buffer (pH 7.4) at 10 mg/mL, then reacted with CF-NHS at 10 nmol/mg for 24 hours. Samples were washed by centrifugation (14,000 rpm, 15 min, 7 cycles) followed by lyophilization process similar to that stated at **2.4.b.i.**

Next, 2 mg of lyophilized fluorescent SFNs were dispersed in 2 mL tris buffer, sonicated, and vortexed to achieve a homogeneous suspension. Then, 200 μ L aliquots were placed in a dark 96-well plate, and fluorescence was measured at 517 nm using a FLUOstar Omega microplate reader. All measurements were performed in triplicate to ensure reproducibility. A simple scheme of the reaction is expressed in figure 8.

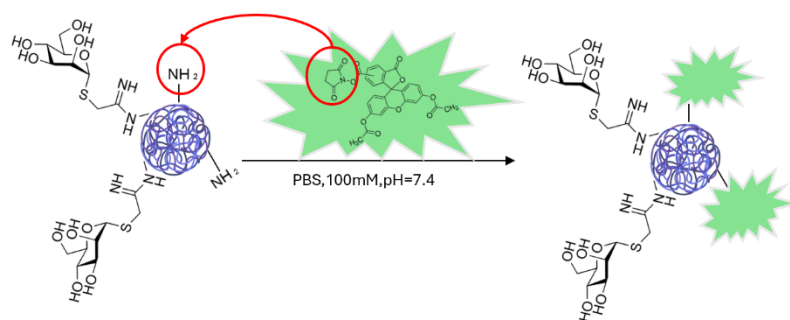


Fig.8. A scheme of reaction between CF-NHS and free amines on glycosylated SFNs (1st Formulation)

2.6.b.1.C/ Azide Flour 545 Fluorescent Dye (Quantifying the triple bond on the surface of glycosylated SFNs):

To quantify the amount of triple bond available on the first formulation. SFNs functionalized with sugar (via the IME approach) and with a triple bond linker were glycosylated with a dye. Specifically, Azide-Flour 545, fluorescent probe linked to an azide group (Azide Fluor 545, MW 631.7 g/mol) in order to simulate the click chemistry with SFNs surface triple bonds (41). A calibration curve was prepared by serial dilution of the dye stock solution (100 nmol/mL) in phosphate buffer, with concentrations of 0.5, 1, 10, 20, and 25 nmol/mL.

2 mg of SFNs with IME sugar (10 and 20 nmol/mg) and triple bond linkers were dispersed in phosphate buffer pH=7.4 (10 mg/mL), sonicated for 2 minutes, and stirred for 30 minutes to equilibrate. CuSO₄ (0.3 eq., 25 nmol/mg) and sodium ascorbate (1.5 eq., 120 nmol/mg) were added, followed by azide-Fluor 545 dye (10 nmol/mg). Reactions proceeded for 2 days under stirring. Washing and lyophilization was performed as described in section **2.4.b.1.B.** Fluorescence measurements at 545 nm were

conducted following the CF-NHS protocol, except phosphate buffer at pH 7.4 was used instead of tris buffer. A simple scheme of the reaction is expressed in figure 9.

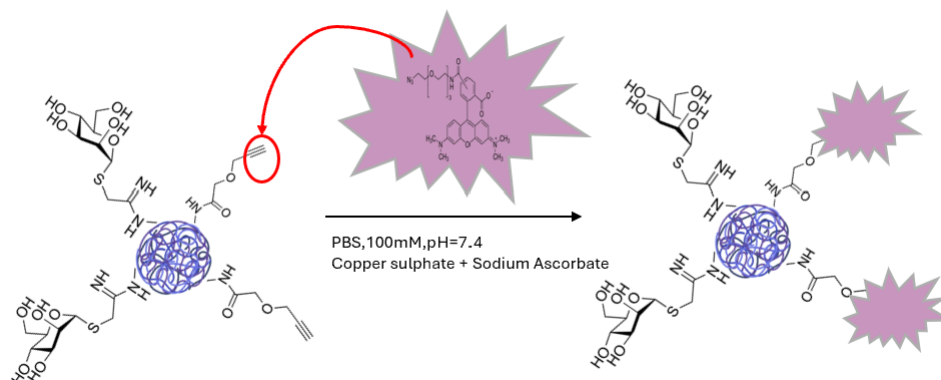


Fig.9. A scheme of reaction between Azide-Fluor 545 and free triple bonds on glycosylated SFNs (1st Formulation)

2.6.b.2. 2nd Formulation

2.6.b.2.A/ Azide Flour 545 dye:

To evaluate the availability of reactive triple bonds on the surface of the SFNs, 5 mg of alkyne-functionalized nanoparticles were reacted with Azide-Fluor 545 dye. To ensure complete coverage of all surface-exposed triple bonds, the reaction was performed with an excess of dye. However, care was taken to avoid dye entrapment, which could lead to misleading quantification. To this end, azide Fluor 545 dye was reacted at concentrations of 10 and 40 nmol per 1 mg of alkyne-functionalized SFNs. In parallel, two samples (each 2 mg) of SFNs previously glycosylated with either M1 azide or M4 azide were reacted with the same dye at 10 nmol of the dye for each 1 mg of the previously glycosylated SFNs with M1 /M4 azide. The glycosylation, washing and lyophilization steps followed the same protocol stated in CF-NHS. The Florescence measurements for the four different formulations were done similar to the protocol stated previously in section 2.6.b.1.C A simple scheme of the reaction is expressed in figure 10

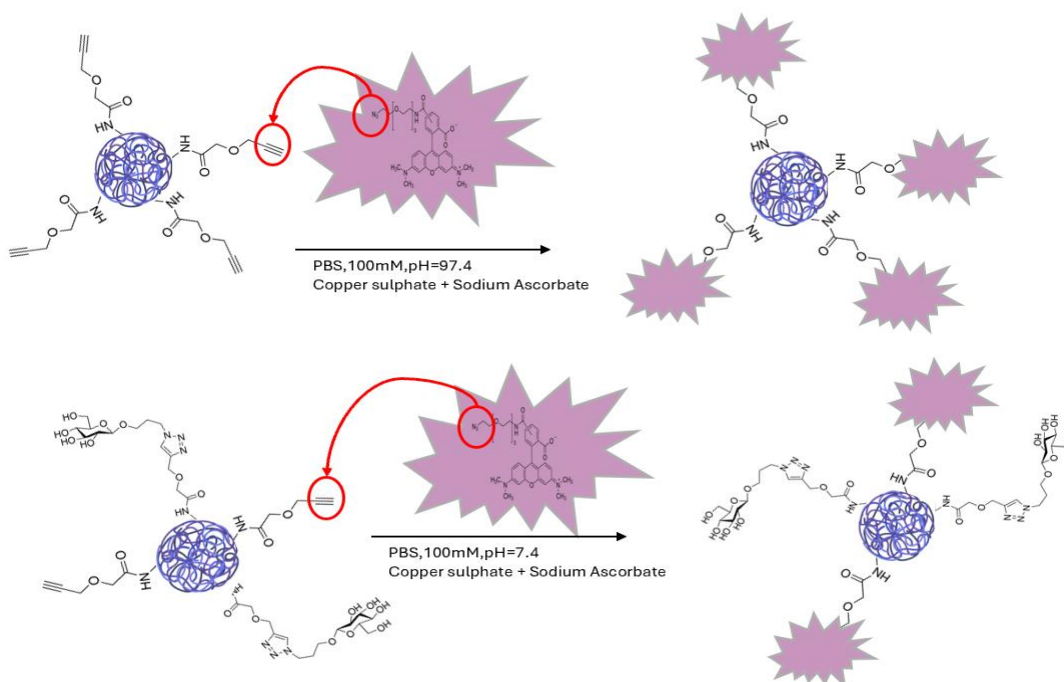


Fig.10. A scheme of reaction between Azide-Fluor 545 and free triple bonds on non & glycosylated SFNs (2nd Formulation)

3.Results and Discussion:

3.1. Organic Synthesis of mannose moieties:

To enable their conjugation, M1-and M4 were functionalized at the anomeric position using suitable chemical linkers—specifically, thiocyanomethyl (a) and azide (b) groups. These modifications allowed for their attachment through two different glycosylation strategies: indiscriminate glycosylation (IME), a mild method, and click chemistry, a highly efficient approach. The objective was to explore whether varying the amount of M1 or M4 incorporated via these methods could enhance cellular internalization through the (MRs) and DC-SIGN. Furthermore, we tried to investigate whether the M4, when linked to SFNs, would exhibit stronger binding affinity to the receptors compared to the M1.

3.2. Quantification of lysine content in blank SFNs:

As previously mentioned, before the start of the glycosylation reactions, it was curial to determine the amount of lysine (primary amines) in 1 mg of the SFNs to adjust the amount of the reactants needed in all the subsequent reaction. According to the literature review, lysine content was estimated to be around 0.3 mol % of the whole protein content (42). Furthermore, very few articles have reported the amount of lysine on the surface of SFNs to be 80 nmol/mg (43). However, Since the amount of lysine in a natural product like silk is dependent on many factors such as the nutrition of the silk worm, so a batch to batch variability is highly expected in our case, so it was essential to conduct this experiment.

It was possible to achieve a calibration curve made by derivatized lysine as shown in figure ... with a good linearity and correlation coefficient $R^2=0.9999$, with a limit of detection = 0.29 nmol/ml and a limit of quantification= 0.88 nmol/ ml. The derivatized lysine had a sharp peak at retention time of 23.5 minutes while the peak of the derivatizing agent L-FDVA eluted earlier at 18.2 min with a complete separation between the two peaks.

While in the chromatogram of the hydrolysate of the protein, the lysine peak in the SFN's chromatogram was small confirming the limited lysine content in SFNs. However, the peak of lysine was well separated from other peaks in the chromatogram. Then for quantification, the AUC of the lysine peak in the hydrolysate was compared to the calibration curve prepared previously and it was found out that this correlates to 46.1 ± 6.70 nmol of lysine/ mg the SFNs.

Furthermore, this total amount of lysine (46.1 ± 6.70 nmol/mg) is present in the whole protein before formulating into a nanoparticle, after which the amount of lysine exposed on the surface of the nanoparticles will be much lower. This experiment was conducted on four different times to confirm the amount of lysine as shown in table 1.

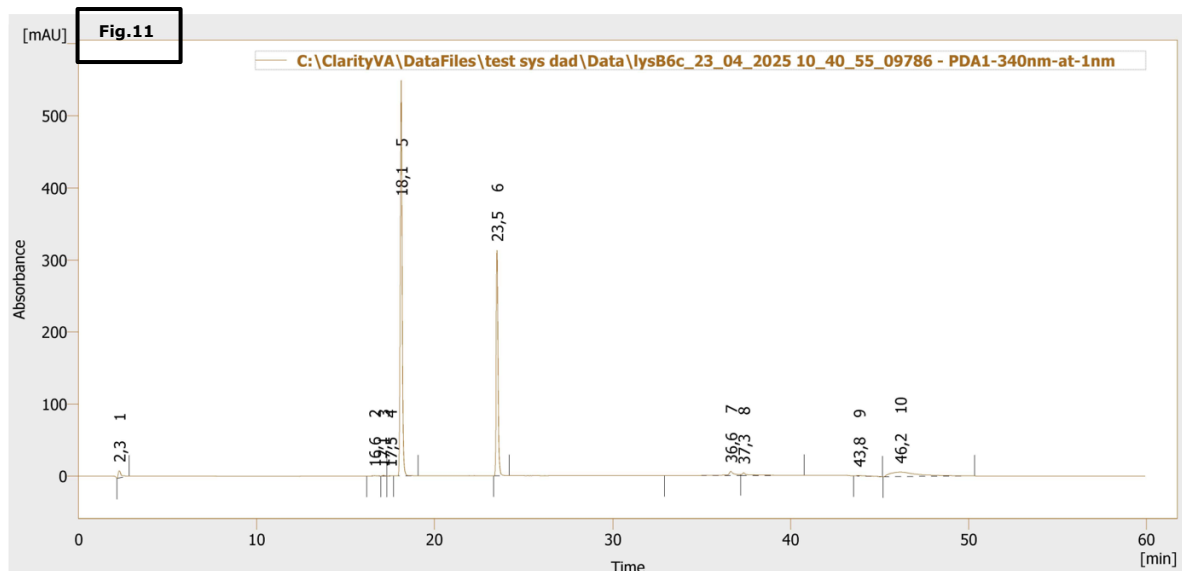
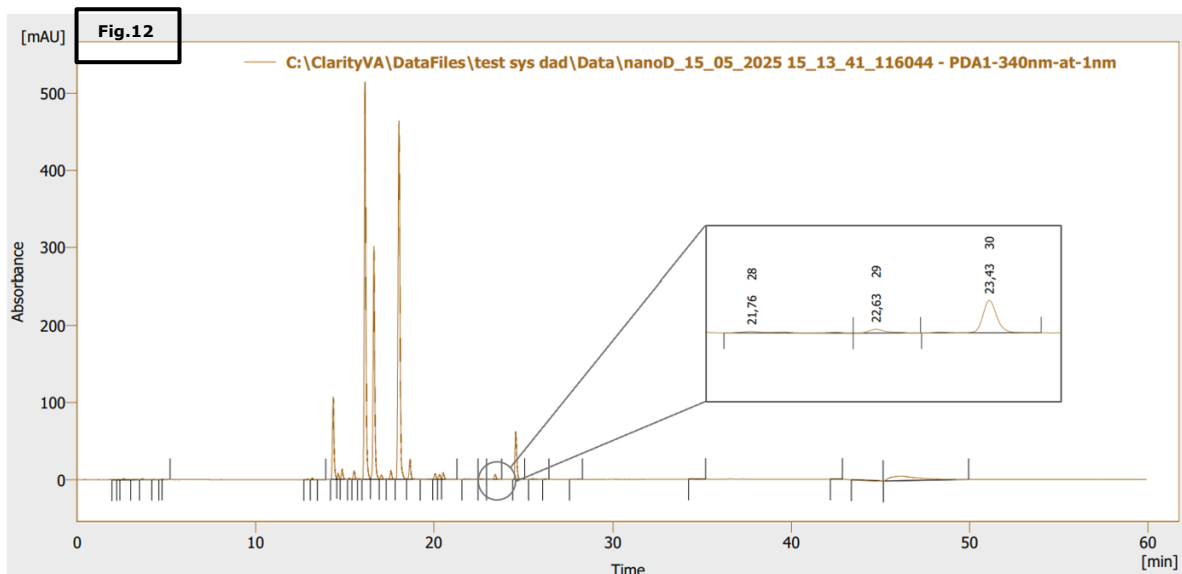


Fig.11. HPLC chromatogram of derivatized Lysine and L-FDVA.

Fig.12. HPLC chromatogram of derivatized hydrolysate of SFNs.

	1 st experiment	2 nd experiment	3 rd experiment	4 th experiment	Average
Lysine content (nmol/mg)	47.47372	39.24778357	42.90375651	54.78566859	46.1 ± 6.70

Table.1, Amount of lysine obtained at four different experiments of SFNs hydrolysate assessment

3.3. Characterization of SFNs:

Particle Size (NTA) and Zeta-potential (DLS) :

The blank SFNs exhibited a mean size of 205 ± 21 nm. However, following functionalization, there was a stepwise increase in the mean particle size at each stage of functionalization. In the first formulation, increasing the amount of incorporated sugar led to a size increase from 251 nm to 299 nm, with the highest values observed when more M.1 was used. The same increase in size was observed when functionalization with M4 was done. A further size increase to approximately 315 nm was observed after

subsequent functionalization with a triple bond linker. Although lyophilization might contribute to the size increase, it is unlikely to be the sole reason, as all samples underwent the same lyophilization process. In the second formulation, particle size also increased after linker attachment. However, the subsequent functionalization with M1 and M4 azide resulted in a less pronounced size increase compared to the first formulation (IME).

For zeta potential, the reconstituted blank SFNs showed a near-neutral charge (0 ± 0.1 mV), as expected due to the presence of unmodified surface groups. In the 1st formulation (IME), after functionalization with different amounts of M1 and M4, the zeta potential shifted slightly to the negative side. This can be attributed to the partial consumption of free amino groups, mainly from lysine residues on the SFN surface, during the conjugation process. The negative charge became more pronounced when a triple bond linker was added, likely due to further reaction with available lysines, leading to reduced surface positivity. In the 2nd formulation (click chemistry), addition of the triple bond alone did not significantly change the surface charge (-0.1 ± 0.2 mV), remaining close to neutrality. However, after conjugation with M1 and M4, a sharp drop in zeta potential was observed. This may be explained by a higher degree of surface modification. Although triazole rings formed during the click reaction are neutral, their polarity could increase surface hydration, contributing to a more negative apparent zeta potential.

As for the difference between M1 and M4 in the click chemistry formulation, the relatively less negative charge in the M4 group might be due to steric hindrance caused by the bulky tetrasaccharide structure, possibly shielding part of the surface and reducing the exposure of the modified areas.

In summary considering these values, glycosylation of the NPs can be qualitatively confirmed in both formulation due to the development of negative charge in both formulations. The values for size and zeta-potential values are summarized in table 2.

SFN		Mean (nm)	Zeta-Potential (mV)
Blank SFNs		205±21	0±0.1
1st Formulation	SFNs M1. (10 nmole/mg)	251±11	-0.9±0.4
	SFNs M1.(20nmole/mg)	265±33	-0.7±0.3
	SFNs M1.(40nmole/mg)	299±39	-0.9±0.2
	SFNs M4. (20nmole/mg)	283±53	-1±0.5
	SFNs M1.(10 nmole/mg) + Triple linker	310±39	-3±0.4
	SFNs M1.(20 nmole/mg) + Triple linker	325±21	-5.6±0.8
2nd Formulation	SFNs + Triple linker	217±24	-0.1±0.2
	SFNs + Triple linker + M1. (4nmole/mg)	226±20	-11.3±0.7
	SFNs + Triple linker + M4. (4nmole/mg)	231±15	-7 ±1.2

Table 2, Characteristics (Size and Zeta-potential) of blank and Glycosylated SFNs.

3.4.Quantification of glycosylation:

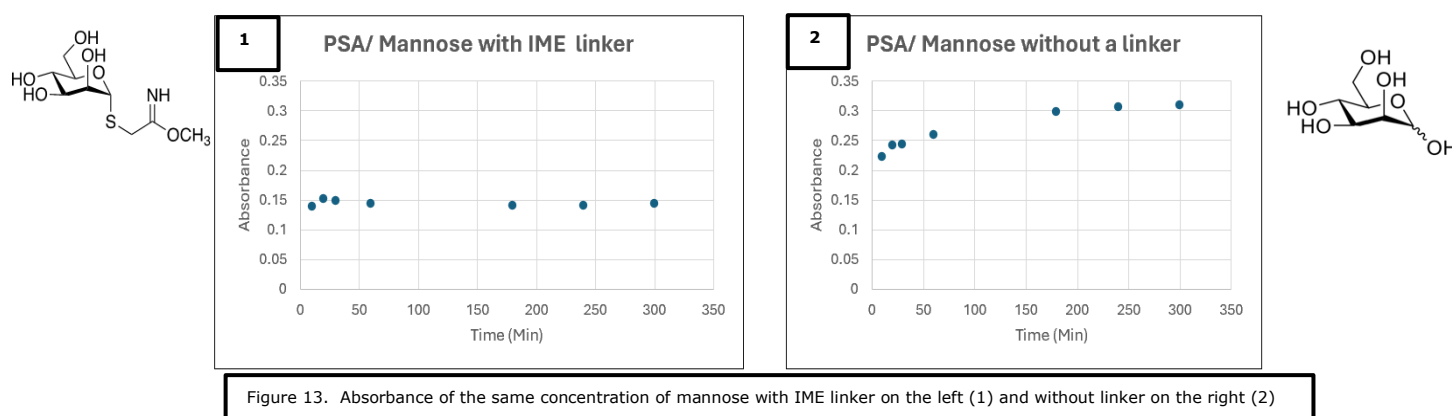
3.4.A.Direct quantification: Phenol Sulfuric Acid (PSA) :

In the beginning, we tried to quantify the glycosylation of mannose directly by using the PSA method for carbohydrate detection. PSA was developed by by DuBios et al (44) for the quantification of sugars.

However, this method has many problems related to the reproducibility of the results and too many optimisation protocols have been reported in the literature (45). It operates through acid-induced hydrolysis of carbohydrates, followed by dehydration to form furfural derivatives, which are detected by absorbance measurement at 490 nm, typically targeting hexoses. While primarily applied for the general analysis of carbohydrate content, this method has also been employed in some studies to estimate surface-bound sugars on nanoparticles (46).

In our investigation, we applied this approach to quantify carbohydrates conjugated to SFNs, using a calibration curve based on Mannose M1 functionalized with an IME linker. However, we found that absorbance values differed depending on the mannose type used. Specifically, the M1-IME conjugate produced a distinct absorbance compared to that of free mannose. Moreover, absorbance measurements taken over a 5-hour period revealed time-dependent discrepancies between the two mannose forms, as illustrated in the corresponding graph, as shown in figure 13.

Furthermore, the absorbance of the blank SFNs was 0.1020 ± 0.0030 for 1 mg, while for 1 mg of SFNs with triple bond linker only it was 0.1667 ± 0.0042 . These observations strongly suggests that the absorbance is not only dependent on the amount of sugar but also the presence and the type of the linker either IME or a triple bond affect the absorbance signal, potentially leading to interfere with the estimation of carbohydrate content. For the beforementioned reasons, we decided to conclude that PSA method was not suitable to quantify the amount of glycosylated content in our case.



3.4.B. Indirect Quantification:

3.4.B.1/ 1st Formulation:

a) O-phthalaldehyde (OPA) assay: Performing the OPA assay on solubilized forms of both blank and glycosylated SFNs enabled the detection and quantification of free, non-glycosylated amino groups through the formation of fluorescent isoindole derivatives. This analysis was conducted to validate results previously obtained using HPLC derivatization by L-FDVA. The lysine content in the blank solubilized SFNs was found to be 40.48 ± 1.08 nmol/mg, which closely aligns with the HPLC-derived value of 46.1 ± 6.70 nmol/mg, determined using a calibration curve generated from a reference protein (bovine thyroglobulin) with a known lysine content.

Applying the same method to glycosylated SFNs—functionalized with varying amounts of sugars—was expected to yield lower levels of free amino groups, since glycosylation typically blocks these reactive sites. By comparing amino group levels before and after glycosylation, the degree of modification could

be inferred. For SFNs glycosylated with M1 at 40 nmol/mg, the remaining free amino content was 38.22 ± 0.94 nmol/mg. Similar values were observed for M1 at 20 and 10 nmol/mg (38.67 ± 0.55 and 38.82 ± 0.84 nmol/mg, respectively). In the case of SFNs functionalized with M4 at 20 nmol/mg, the result was 38.59 ± 0.62 nmol/mg. From these differences, the estimated levels of glycosylation were:

- M1.a at 10, 20, and 40 nmol/mg: 1.66, 1.81, and 2.26 nmol/mg respectively
- M4.a at 20 nmol/mg: 1.89 nmol/mg

These findings suggest that the tetrasaccharide (M4) reacts similarly to the monosaccharide (M1) under equivalent conditions. The relatively modest reduction in free lysines is consistent with expectations, as only about 10–20% of total lysine residues are typically surface-accessible and available for modification. All values are summarized in table 3.

Solubilized NPs	Reaction conditions	Free Amine	Glycosylated sugar
Blank SFNs	-	40.48 ± 1.08	-
SFN M1	10 nmol/mg	38.82 ± 0.84	1.66 nmol/mg
SFN M1	20 nmol/mg	38.67 ± 0.55	1.81 nmol/mg
SFN M1	40 nmol/mg	38.22 ± 0.94	2.26 nmol/mg
SFN M4	20 nmol/mg	38.59 ± 0.62	1.89 nmol/mg

Table 3, OPA method-glycosylation results of blank and glycosylated SFNs with different quantities.

b) 5(6)-Carboxyfluorescein *N*-hydroxysuccinimide ester dye

Since the primary objective of this project was to functionalize SFNs with both the targeting moieties (M1 and M4) and an antigenic peptide. It was an essential step to prove and quantify the amount of primary amine groups that are still accessible on the surface of the SFNs and can be functionalized in the following steps.

To achieve this, (CF-NHS) ester dye was used. This dye selectively reacts with free amine groups, forming a fluorescent product detectable by fluorimetry at 517 nm. A standard calibration curve was established over a concentration range of 0.5–10 nmol/mL, yielding a strong linear correlation.

Using this method, the quantity of dye bound to glycosylated SFNs was measured. For SFNs glycosylated with 10 nmol/mg and 20 nmol/mg of sugar, the amount of dye conjugated was found to be 2.368 and 1.958 nmol/mg, respectively. When these results were compared with those obtained from the OPA assay for the same formulations, the total number of accessible lysine residues available for functionalization was estimated to be approximately 4 nmol/mg.

c) Azide Flour 545:

It was possible to obtain a Linear relationship for the calibration curve of the dye with a good correlation coefficient $R^2=0.9921$ for the dilution points (0.5-25 nanomole per/ml). As Shown in the table..., Following that we introduced a linker with a triple bond to the free amine groups which are functionalized with a M1 at (10 & 20 nmol/mg) in order to maximize the possibility if attachment of the peptide. Followed by attaching the azido flour 545 at a conc (10 nmol/mg) to quantify how many peptides can be hypothetically attached into each case. The results have shown that the amount of dye that can be

attached are nearly similar = 1 nmol/mg . This result can be justified by the fact that we performed two consecutive reactions on the amino groups on the SFNs and it's not possible to modify all of them to a triple bond linker. Furthermore, the steric hindrance can also play a role in reducing the amount of lysine that are accessible for the reaction with the dye on the surface.

3.4.B.2 / 2nd Formulation:

Azide Flour 545:

Given that the functionalization strategy in this formulation relies primarily on the reaction between alkyne functionalized SFNs and azide-functionalized mannose moieties, it was critical to quantify the number of accessible triple bonds on the surface of the SFNs. To achieve this, both non glycosylated, Alkyne functionalized SFNs and SFNs pre-glycosylated with sugars (M1 and M4., at 4 nmol/mg) were subjected to dye labeling. The primary objective was to determine the total number of reactive linkers available on the SFNs' surface and, subsequently, to estimate the degree of glycosylation by calculating the reduction in available linkers following sugar conjugation.

Incorporation of a fluorescent dye into 1 mg of alkyne-functionalized SFNs (bearing only triple bonds) reached 3.842 ± 0.039 nmol when reacted with the dye at a concentration of 40 nmol/mg. When the dye concentration was reduced to 10 nmol/mg, the incorporation efficiency decreased to 2.159 ± 0.029 nmol. In contrast, SFNs pre-functionalized with the sugars M1. and M4 demonstrated lower dye incorporation, yielding 1.159 ± 0.028 and 1.183 ± 0.035 nmol, respectively. These results correspond to the successful glycosylation of approximately 2.683 ± 0.048 and 2.659 ± 0.052 nmol of M1. and M4., respectively, per 1 mg of SFNs. These values were compared to the maximum dye incorporation (3.842 ± 0.039) observed in non-glycosylated SFNs, likely reflecting near-complete utilization of all available surface triple bonds. Additionally, the more negative zeta potential values observed in M1 and M4-conjugated SFNs (Formulation 2 click chemistry) compared to the formulation 1 (IME approach) indicate enhanced surface modification. This is consistent with the high efficiency of click chemistry for glycosylation. Furthermore, the lower dye incorporation at 10 nmol/mg in sugar-functionalized SFNs supports the hypothesis that reduced dye loading minimizes the chance of nonspecific dye entrapment.

4. Conclusion and Future Perspectives:

In this study, the lysine content in 1 mg of SFNs was successfully determined to be approximately 40 nmol/mg of total protein using two methods: OPA and HPLC derivatization with L-FDVA. Different amounts of sugars M1 and M4 were then incorporated using two chemistries—IME and click chemistry. Direct assessment of glycosylation by the PSA method was not feasible due to linker interference. However, glycosylation was qualitatively indicated by an increase in particle size in both formulations especially 1st formulation and a shift to a more negative surface charge, likely due to lysine consumption—(an effect more pronounced with the click chemistry method). Consequently, glycosylation was assessed indirectly using the OPA method, CF-NHS dye, and Azide-Fluor 545 dye. For the first formulation (IME), reaction of SFNs with 10 and 20 nmol/mg of M1. sugar resulted in functionalization of 1.6 and 1.8 nmol/mg, respectively, leaving 2.368 and 1.958 nmol/mg of free lysine . This suggests

that approximately 4 nmol/mg of lysine is surface-accessible which was further confirmed by the results of the second formulation. For M4.a, reaction at a 20 nmol/mg ratio led to glycosylation comparable to M1, at 1.89 nmol/mg. In the second formulation (click chemistry), the triple bonds on SFNs measured 3.841 nmol/mg. After glycosylation with 4 nmol/mg of M1. or M4., 2.6 nmol/mg was incorporated, leaving approximately 1.2 nmol/mg of lysine available for the attachment of the antigenic peptide.

Finally, we successfully demonstrated the feasibility of heterofunctionalizing SFNs using two distinct strategies, enabling the simultaneous attachment of both targeting ligands and antigenic peptides to the same SFNs. To the best of our knowledge, this is the first comprehensive quantification conducted specifically for SFNs, establishing a robust reference framework for future comparative analyses.

As future prospectives, it will be essential to assess whether the specific amounts of M1 and M4 conjugated to SFNs are sufficient to enhance binding to MRs and DC-SIGN, in comparison to unmodified SFNs. Although this enhancement has been well established in soluble proteins such as albumin, its effectiveness following surface functionalization to nanoparticles remains to be demonstrated. Surface plasmon resonance (SPR) analysis could be a suitable tool to quantify these interactions. Furthermore, if one of the mannose derivatives exhibits superior binding at the tested concentration, it will be important to investigate whether this correlates with improved uptake by APCs, thereby confirming the biological relevance of the receptor targeting strategy.

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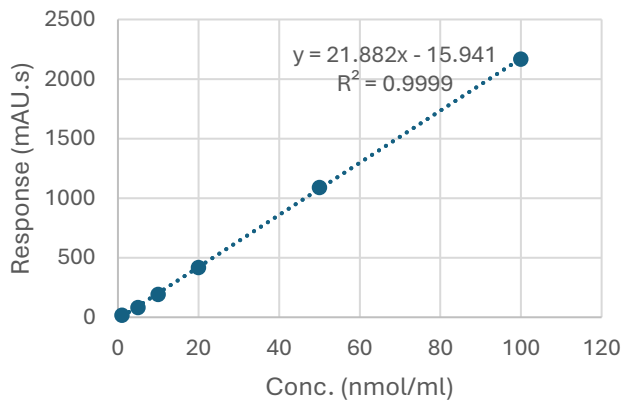
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Annex

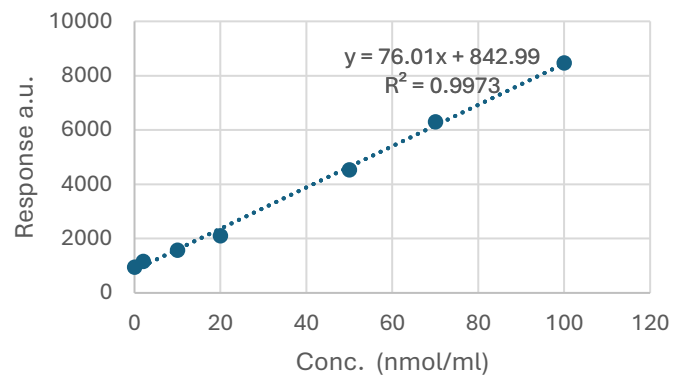
1

Lysine Calibration Curve



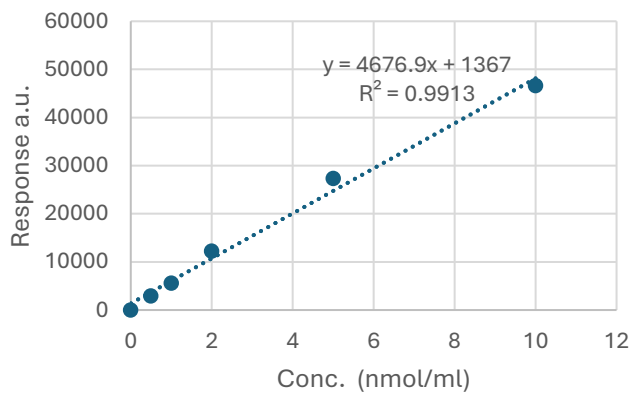
2

Thyroglobulin



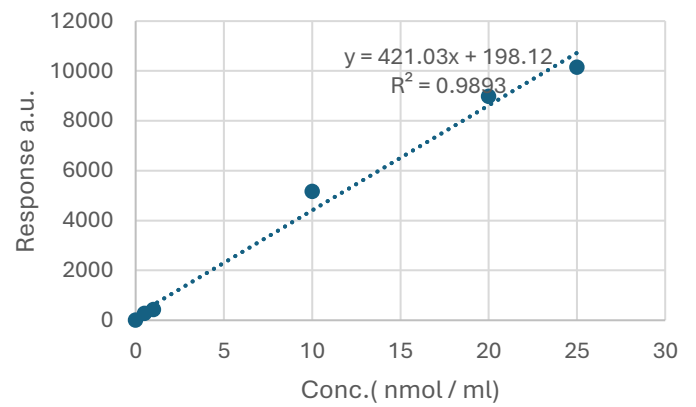
3

5(6) Carboxy Florescin NHS ester



4

Azide-Flour 545



Abstract

Tuberculosis (TB) remains the world's leading cause of death from a single infectious disease, according to the 2023 WHO report. TB is caused by *Mycobacterium tuberculosis* (MTB), primarily affecting the lungs. While Bacillus Calmette–Guérin (BCG) is the only approved vaccine against TB and can induce CD4⁺ T cell memory and a phenomenon known as "trained immunity"—which enhances immune responses to unrelated pathogens—its protective efficacy diminishes with age. Consequently, the majority of new TB cases occur in individuals older than 15 years. Moreover, additional booster doses of BCG have shown limited to no improvement in immune protection.

To address these limitations, alternative vaccine strategies are being explored, including whole-cell vaccines, viral-vectored vaccines, and subunit vaccines. Our work focuses on developing a subunit vaccine utilizing nanocarriers to enhance antigen delivery and immune response. Specifically, we use silk fibroin nanoparticles (SFNs) due to their known biocompatibility, biodegradability, and intrinsic adjuvant properties that can help overcome the typically poor immunogenicity of subunit vaccines.

To target antigen-presenting cells (APCs) via mannose receptors (MR) and DC-SIGN, two mannose derivatives (M1 and M4) were synthesized and conjugated to SFNs using indiscriminate glycosylation (IME) and click chemistry. We aimed to investigate whether varying sugar densities and structural types affect receptor binding and cellular uptake. The lysine content in SFNs was determined to be 46.1 ± 6.70 nmol/mg, consistent with OPA assay results of 40.48 ± 1.08 nmol/mg, providing a basis for subsequent functionalization.

We employed two chemical strategies for SFN glycosylation: (1) an indiscriminate glycosylation method (IME) and (2) a click chemistry-based approach.

In both formulations, glycosylation led to an increase in nanoparticle size, possibly proportional to the amount of sugar used. Additionally, a shift in zeta potential toward a more negative charge was observed following glycosylation. This shift qualitatively supports the success of glycosylation, as it indicates the consumption of surface amine groups, reducing their positive charge, during sugar conjugation.

In the first formulation (IME), glycosylation with M1 at concentrations of 10, 20, and 40 nmol/mg yielded incorporation levels of 1.6, 1.8, and 2.2 nmol/mg, respectively. Similar glycosylation levels were achieved with M4 (1.89 nmol/mg at 20 nmol/mg). These modifications left approximately 2 nmol/mg of lysine available for peptide attachment. In the second formulation (click chemistry), triple bonds on SFNs were quantified at 3.841 nmol/mg. After glycosylation with 4 nmol/mg of M1 or M4, 2.65 nmol/mg was incorporated, leaving ~ 1.2 nmol/mg of lysine for antigen conjugation.

Future studies, including surface plasmon resonance (SPR) and cellular uptake assays, are planned to evaluate how sugar density and spatial configuration influence receptor binding and APC internalization.

Keywords: Silk Fibroin Nanoparticles (SFNs), Tuberculosis (TB), Subunit Vaccines, Mannosylation, Targeting Mannose receptors (MR).