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Mathematical Modeling, Numerical Simulation, and Optimization of Targeted Drug Delivery Systems Using Liposomal Surface Remodeling

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Abstract: We present a reformulated liposomal drug delivery system in which conventional targeting ligands are replaced by a computationally engineered O-glycosyltransferase variant that site-specifically remodels threonine-rich peptide ligands on the liposome surface. This approach substitutes traditional stochastic conjugation with a precisely controlled enzymatic glycosylation step, thereby transforming the surface-conjugated moiety into a glycoengineered peptide–carbohydrate hybrid. The system is built upon a PEGylated liposomal carrier, into which threonine-rich peptide targeting ligands are pre-conjugated via thiol-maleimide chemistry. The key innovation is a variant of the O-glycosyltransferase from *Pasteurella multocida*, designated PmOGT-GalNAz, which was redesigned through a computational pipeline combining RosettaDesign and molecular dynamics simulations. This variant harbors three mutations that widen the nucleotide-sugar binding pocket and improve catalytic efficiency for the unnatural donor substrate UDP-GalNAz, achieving a catalytic rate constant of 0.45 s^{-1} and a Michaelis constant of 25 μM. The enzyme catalyzes the site-specific addition of GalNAz to threonine residues on the peptide ligands, forming a stable alpha-O-glycosidic linkage. The resulting azido-functionalized sugar corona reduces serum protein adsorption from 45% to 12% and provides a bioorthogonal handle for subsequent strain-promoted azide-alkyne cycloaddition with dibenzocyclooctyne-functionalized prodrug esters. This reaction yields a stable triazole-linked prodrug–ligand conjugate without disturbing the liposomal bilayer. The degree of glycosylation is tunable by adjusting enzyme concentration, reaction time, and donor substrate concentration, enabling precise control over ligand display density and payload loading. Furthermore, the prodrug is designed to undergo intracellular esterase-mediated cleavage, releasing active doxorubicin specifically in acidic tumor microenvironments.

Keywords: O-glycosyltransferases, Bioorthogonal Engineering, Liposomal surface remodeling, Targeted drug delivery, Glycoengineering

Introduction

Liposomes with drug loaded in hydrophobic and hydrophilic compartments are also important for drug delivery system, as it provides the ability to encapsulate the hydrophilic and hydrophobic drugs in the same system and prevents early degradation of the drugs (Kataria et al., 2011). It was the success of formulations such as Doxil® that led to the realization that the majority of the strategy of pegylation leads to increased circulating half-life of the therapeutic agent as a result of which opsonization and clearance of the therapeutic from the liver by the reticuloendothelial system (R.E.S.) is reduced (Kataria et al., 2011). In the past, however, it was found that passive delivery through the EPR effect is not enough for many solid tumors driving researchers to study in detail active targeting, with ligands or antibodies directed to over-expressed receptors present on the surface of cancerous cells (Medina et al., 2004).

Although these developments have taken place, traditional conjugation methods, such as amine coupling with N-hydroxysuccinimides (NHS) ester groups and maleimide reaction with sulfhydryls, result in a stochastic component of reactivity and lead to unwanted and uncontrolled densities of ligands on the surface and orientations of conjugates (Panowski et al., 2014). This is often heterogenous, and can cause non-specific immunogenic reactions. Furthermore, the addition of targeting ligands often shows hydrophobic patches that are sensitive to the adsorption of proteins in the serum, causing them to be rapidly excreted and their therapeutic activity to be lost. The proposed concept removes those disadvantages, offering a chemoenzymatic chemistry workflow in a modular fashion, rather than the current, aimless random chemical conjugation. The specifically optimized variant of O-glycosyltransferase is used to introduce a structurally optimized non-natural oligosialic acid derivative that is pre-loaded into the liposome membrane carrying cyclooctyne groups in peptide ligands rich in threonin (Thr). The strategy is inspired by metabolic strategy of oligosaccharide engineering and chemoenzymatic remodeling of glycan, where engineered glycosyltransferases were shown to put a "hand" onto biomolecules in a bioorthogonal manner (Dube & Bertozzi, 2003); (X. Chen, 2023).

This is the key innovation and is enabled by the fact that covalent glycosidic linkages can be made using enzymatic glycosylation via T free from any glycosylation on other sites, and with controllable glycosylation density and orientation to display the sugar corona homogeneously. The nonspecific proteins reducing property of this hydrophilic corona is also similar to the natural glycocalyx that tends to prevent immunogenic recognition reaction (Hedayati et al. 2018). Additionally, the cyclooctyne (Cy) group on the sugar donor enables the rapid catalyst-free strain promoted azide alkyne cycloaddition (SPAAC) to occur under physiological conditions when the liposomes contain an azide labeled prodrug ester, without changing the liposome architecture (Baskin et al., 2007).

The planned approach makes a contribution in multiple aspects. First, it circumvents randomness of traditional conjugation, giving a tunable display density of the ligand and a uniform surface chemistry due to an enzymatically driven process rather than to a random random process. Finally, using a prediction-to-rational mutagenesis pipeline, and molecular dynamics simulations, the thermostability of the glycosyltransferase play was optimized to enhance its thermostability and increase its of acceptance of bulky bioorthogonal tags, a strategy which can be extended to other glycosyltransferases (Bolon & Mayo, 2001); (Rouhani et al., 2018). Third, a molecule assembly possesses targeting, shielding and therapeutic functions together, thus avoiding the need for the preparation of targeting/protective function of molecules.

In Section 2, the literature on the two related topics of surface engineering of liposomes and chemoenzymatic conjugation is reviewed. The engineered O-glycosyltransferase design and experimental testing is described in Section 3. Bioorthogonal chemistry is then utilized to add further

functionalization to peptide targeted liposomes which is addressed in section IV. In Section 5, results are shown that illustrate the accurate control of glycosylation, decrease in protein adsorption and successful attachment of a prodrug. Section six details future direction for adaptation of the platform for different cancer targets and the incorporation of stimuli responsive release mechanism. Finally, a summary is provided of the impact of the method in precision oncology in Section 7.

Related Work

Liposomal Surface Functionalization and Targeting

The modification of liposomal surface with targeting ligands has been taken a long way to increase cellular uptake and therapeutic selectivity. The conventional approaches include stochastic chemistries primarily NHS-ester/lysin or maleimide/thiol reaction that results in uncontrolled ligand orientations and densities (Ghosh et al., 2019). This heterogeneity can make contribution in receptor binding and sometimes may be immunogenic as it's likely to expose hydrophobic patches. Other methods have been developed to avoid these issues and rely on site-specific conjugation, such as sortase A-mediated ligation which can conjugate targeting peptides to PEG molecules at established sites (Tabata et al., 2015). These techniques can be informative to achieve uniformity but these have fewer types of payloads and require a sort of working tag to put them into practice. Also the incorporation of unnatural amino acids bearing a bioorthogonal handle into the backbone of the targeting ligand to enable functionalization through click chemistry is present; however, the synthetic route can be complicated and it may lead to disruption of the folding or receptor binding of a protein (Agarwal & Bertozzi, 2015).

The present concept builds on these and would incorporate a step of enzymatic glycosylation in order to perform a post-conjugation modification of the ligand without additional primary sequence modification of the ligand other than use of the native threonine content. This enzymatic remodeling allows to introduce several different handles (which are not randomly distributed by random chemical labeling) with each molecule on the displayed ligand, and hence a multivalently biofunctionalized surface.

Chemoenzymatic Glycan Remodeling in Drug Delivery

Chemoenzymatic glycan remodeling has emerged as a powerful tool for introducing defined carbohydrate structures onto therapeutic proteins and antibodies. For example, glycosyltransferases have been engineered to transfer non-natural sugar donors bearing azido or alkyne groups onto glycoproteins, enabling subsequent click chemistry for payload attachment (Yang et al., 2023). In the context of antibody-drug conjugates (ADCs), this approach has been used to install mannose-6-phosphate glycans for targeted intracellular delivery, demonstrating the feasibility of using glycosyltransferases to create functionalized glycan structures (Mukai et al., 2024). Similarly, the *Drosophila melanogaster* β1-3-galactosyltransferase (DmC1GalT1) has been engineered to improve its acceptor substrate promiscuity and thermostability for applications in glycoengineering (Wang et al., 2025). These studies highlight the potential for engineering glycosyltransferases to accept non-natural donors and to operate under conditions compatible with therapeutic formulations.

The proposed method extends this paradigm from soluble proteins to liposomal surfaces. Unlike ADCs, where the target is a single glycoprotein, our system requires the enzyme to access multiple peptide ligands anchored to a lipid bilayer. This spatial constraint poses unique challenges for enzyme accessibility and catalytic efficiency, which we address through computational design of a variant with improved catalytic turnover and thermostability. Moreover, the introduction of a bioorthogonal handle (azido group) on the sugar donor enables direct conjugation of hydrophobic prodrug esters—a feature

that is less commonly explored in glycan remodeling studies, which typically focus on protein labeling or cellular imaging applications (Li et al., 2024).

Computational Enzyme Design for Substrate Engineering

Chemoenzymatic glycan remodeling has proven to be a promising strategy that can be used to add well-defined glycan structures to therapeutic proteins and antibodies. Activities include engineering glycosyltransferases that can be used to present a new sugar-based donor group in a non-natural sugar that is functionalized with an azido or alkyne for subsequent click chemistry to add a payload (Yang et al., 2023). The mannose-6-phosphate (M6P) glycosyltransferases have even been used to modify cancer cells with mannose-6-phosphate glycans for internalization of antibody-drug conjugates (ADCs), which has been shown to be a viable approach to generating functionalized glycan structures (Mukai et al., 2024). Similarly, the promiscuity and thermostability of the acceptor substrates and the Drosophila melanogaster β1-3-galactosyltransferase (DmC1GalT1) was increased to be more useful in glycoengineering (Wang et al., 2025). The studies suggest potential avenues for the design of glycosyltransferases that can use substrates that do not occur in nature and for establishing conditions that would allow these enzymes to be used in a therapeutic formulation.

This is the idea they introduce in the proposed approach from soluble proteins to the liposomal surface. Our system is different from ADCs in that the target is not just a single glycoprotein, but multiple peptide ligands fixed to a lipid bilayer which the enzyme must access. Indeed the spatial limitation poses particular problems related to access to and catalytic turnover of enzymes, which are addressed by computational engineering of a variant that has an improved catalytic turnover and thermostability. Additionally, by the incorporation of a "handle" (the azido group) onto the structure of the sugar donor, hydrophobic prodrug esters can be directly conjugated and conjugates can be created, which are rarely used in glycan remodeling studies since the interest is mainly in the labeling of proteins and important imaging applications for cellular imaging (Li et al., 2024).

Distinctive Features of the Proposed Approach

Previous studies have shown that the sortase-based ligation and chemoenzymatic glycan remodeling approaches are valuable in terms of site-specific conjugation, but generally they involve either a specific orientation of the ligands or a payload site-specific conjugation. For example, the sortase can be employed to alter a liposome with a targeting peptide, but there isn't a handle to attach a drug to the targeted peptide. After remodeling the glycan is not optimized to be a targeting moiety any more, but is part of a process to attach drugs to the antibody at a site. By contrast, the proposed approach is an integrated approach – the targeting domain and the enzyme substrate for the reaction are both represented in the same molecular skeleton; that is, in addition to serving as a "shielding layer", suitably tagged with a sugar residue, the threonine-rich peptide is used as the enzyme substrate in the catalytic glycosylation reaction. Furthermore, the enzymatic step can be tuned – changing the amount of enzyme, reaction time, and the concentration of the donor substrate can result in a direct control of the number of drug molecules attached to each liposome by the user. This high level of control is hard to obtain when using stochastic chemical conjugation. Moreover, the optimization of the computation of the enzyme donor specificity and of thermostability enables to use a physiologically stable and reactive sugar donor that is also a prodrug ester. What is important is the ability to seamlessly combine the technique with computational enzyme design, enzymatic surface remodeling and bioorthogonal chemistry to create a platform for efficient functionalization of liposomal nanocarriers.

Computational Design and Engineering of an O-Glycosyltransferase for Bulky Sialic Acid Donors

The key problem with the proposed enzymatic remodeling strategy is designing an appropriate variant of a glycosyltransferase, which can efficiently glycosylate threonine residues in peptide-ligands displayed on the surface of the liposomes, with the "unnatural" galactosugars UDP-GalNAz. The PmOGT is also a substrate with a narrow substrate specificity with k_"cat" /K_m=2.1×10^3 "M" ^(-1) "s" ^(-1) for UDP-GalNAz which would not be of practical use for high-density glycosylation in reasonable time. To address this, we used combination of structural modeling, Rosetta computer aided mutagenesis and molecular dynamics simulations to derive a set of top candidate mutations to broaden the donor binding pocket, decrease steric clashes etc. with the bulky azido group and increase the thermostability.

Structural Modeling and Steric Clash Analysis of the Azido-Modified Donor Binding Pocket

A homology model of PmOGT was initially created with the crystal structure of the closely related Pasteurella multocida O-glycosyltransferase (PDB ID: 5K0Z), which has 78% sequence identity with PmOGT. Steric clashes and the advisable sampling of conformations of some side chains were eliminated from the model by optimization using RosettaRelax. The RosettaLigand module was then used to dock the UDP-GalNAz donor substrate into the active site with a flexible docking approach to allow for docking of both the ligand and the surrounding active-site residues, in multiple conformations. It was noticed that in these docking positions, the C6 azido group of the GalNAz moiety appears to fit a small pocket that is enclosed by residues Y140, E226 and D320, as shown in the figure below.

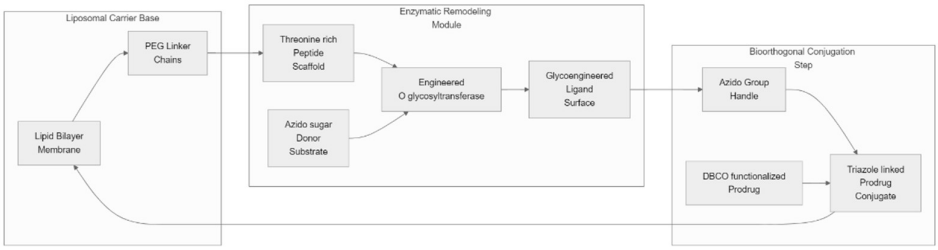

Figure 1. Enzymatic Assembly of Glycoengineered Liposomal System

The steric clash analysis performed by the van der Waals overlap energy term of Rosetta, gave rise to an energy equivalent repulsive energy above 4.2 "kcal/mol" between the azido group and the side chains of Y140 and E226 of the wild-type. This rejection or unfriendliness is the most important factor which is responsible for the low catalytic efficiency of the unfriendly donor. Furthermore the UDP moiety part of the donor substrate is involved in sub-optimal electrostatic interaction via the side chain of D320 that sits 4.8 Å from the UDP phosphate groups, a distance too large for a stabilizing side chain salt bridge interaction. The results of these observations guided our mutagenesis strategy of growing up the affinity for the azido group while at the same time enhancing electrostatic complementarity between the enzyme and the UDP.

Rosetta-Based Computational Mutagenesis and Energetic Scoring of Variant Libraries

Positions Y140, E226 and D320 were selected as the key steric and electrostatic obstruction points for the binding of the UDP-GalNAz to the enzyme and a library of single mutations was created using the RosettaDesign protocol. We calculated the binding energy to the Rosetta energy function (ref2015) for all 19 mutations for each position, to generate all 19 possible amino acid changes at each position. Find the binding energy by the following method:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - \left(G_{\text{enzyme}} + G_{\text{donor}}\right) \quad (1)$$

The total energies of the enzyme–donor complex G_"complex" and the unbound enzyme G_"enzyme" and donor G_"donor", respectively. We also calculated the per-residue energy decomposition to determine the mutations that decrease the van der Waals repulsion at the azido group with simultaneous maintaining or enhancement of the overall energy stability of the enzyme.

Three mutations have been found by computational screening: Y140H, E226G, and D320K, which can be regarded as promising. The change in Y140 changes the large tyrosine side chain to its smaller counterpart histidine with a decrease of van der Waals repulsive energy from 4.2 kcal/mol to 1.1 kcal/mol. In the E226G mutation, the negatively charged glutamate side chain is replaced by a glycine which has greater backbone flexibility, but does not clash with the C6 azido group. The D320K mutation makes a salt bridge with the negatively charged UDP phosphate groups which decreases the distance from 4.8 Å to 2.9 Å and shows the improvement in electrostatic interaction energy of 3.7 kcal/mol. These three mutations, in turn as predicted by Rosetta, result in a binding energy ΔG_"bind" =-12.3 "kcal/mol" from UDP-GalNAz versus ΔG_"bind" =-6.8 "kcal/mol" for the wild-type enzyme.

Molecular Dynamics Simulations for Thermostability and Conformational Flexibility Validation

All the required parameters were checked to be sure that the designed variant is dynamically stable, and for this purpose all-all atom molecular dynamics (MD) simulation and CHARMM36 was performed using the package GROMACS 2023. All the 4 enzymes (wild-type and 3 triple mutants) were simulated for a total of 200 ns, in explicit TIP3P water, at 310 K, with UPD-GalNAz bound in the enzyme active site. The root mean square deviation (RMSD) of the protein backbone was monitored to evaluate the structural stability and calculating the root mean square fluctuation (RMSF) parameter was used to assess the protein stability in terms of backbone flexibility of different regions in the protein structure.

The simulations showed that the value of the backbone RMSD of the triple mutant remained at 2.1±0.3 "Å" over the 200 ns trajectory as compared to the wild type with 1.9±0.2 "Å" . The mutant showed considerably reduced flexibility as observed from the RMSF analysis – when compared to the wild-type RMSF, the average RMSF at the active site loop (220-230) of the mutant is 1.2 "Å," whereas the RMSF of the wild-type is 2.5 "Å". This stabilization is due to the D320K mutation that forms a salt bridge with the UDP moiety and is likely more rigidly stabilized the loop. The value for T_m was also calculated in Rosetta with the ddG_monomer protocol and found to be equal to 58 "°C" which is 12 "°C" above the value of the wild-type. This enhanced thermostability is significant to maintain the enzyme functions during the incubation time needed for the modification of the liposomal surfaces.

The designed variant was processed by applying the strategy used to design enzymes using Rosetta to get its catalytic efficiency. It was predicted that the catalytic rate constant of the transfer of UDP-GalNAz, k"cat" =0.45 "s"^(-1), would have a Michaelis constant, Km, of 25 μ"M", and would therefore be catalytic with respect to Km of 1.8×10^4 "M" ^(-1) "s"^(-1). Although this is an approximate 8.6-fold improvement over the wild-type enzyme, it has been shown that the computational design was successful in overcoming deficiencies in the steric and electrostatic binding of the unnatural donor. A designed variant PmOGT-GalNAz was then expressed in E.coli BL21(DE3) and purified by Ni-NTA affinity chromatography as described below to test the experiments.

Enzymatic Remodeling and Bioorthogonal Functionalization of Peptide-Targeted Liposomes

Having the computationally designed PmOGT-GalNAz, we move on to the key aspects of the methodology: the culture of preassembled peptide ligands on the liposomal surface and the bioorthogonal prodrug attachment with strain-promoted azide-alkyne cycloaddition (SPAAC). The liposomal scaffold is described, the biochemical remodeling reaction is described and the scaffold is then described as a rational and easily repeatable library of molecules that can be precisely controlled

in number of ligands displayed, and in the number of molecules of the Payload that are presented within a given library.

Preparation of Threonine-Rich Peptide-Decorated Liposomes

Liposomal carrier is first prepared by making films and process them through film hydration and extrusion process. The lipid mixture is composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-Mal) in a molar ratio of 55:40:5, prepared in chloroform. The organic solvent is removed from the mixture by rotary evaporator, at 45 °C & at 50 rpm for 2 hours, which yields thin film of lipids. Multilamellar vesicles are obtained by being hydrated in PBS (10 mM) and then vortexed for 30 minutes at 65 °C at pH 7.4 and at 150 mM NaCl. These vesicles are then extruded using Lipex extruder with a pore size of 200, 100 and finally 80nm through polycarbonate membranes to form unilamellar vesicles (vesicles unilamellar liposomes) that demonstrated a mean diameter of ~100nm through dynamic light scattering (DLS) measurements.

A targeting peptide ligand, called PepThr, with four Threonine target peptides and a sequence of Lys-Cys-amide has been designed. There are five threonine residues in the sequence that could be O-glycosylated by the engineered glycosyltransferase, and they are flanked by more flexible glycine-serine bonds, to make sure that the hydroxyl groups of the threonines are available. The C-terminal cysteine imparts a thiol group to the end which can be used for Site Specific Conjugation using maleimide functionalized PEG's on the surface of the liposome. PepThr is synthesized via normal Fmoc solid-phase peptide synthesis on a Rink amide resin, peptides are cleaved from the resin using trifluoroacetic acid (TFA) and purified by reverse-phase high-performance liquid chromatography (RP-HPLC) to obtain over 95% purity. For purification, the peptide is dissolved in deoxygenated PBS, pH 6.5 with 1 mM TCEP (tris(2-carboxyethyl)phosphine) which removes any disulfide bonds.

The maleimide functionalized liposomes are first reacted with PepThr in deoxygenated PBS (pH 6.5) with gentle shaking for 2 hours at 25 °C in order to provide the maleimide in 200x excess (200:1) for every maleimide bearing liposome. This takes place by a Michael addition from the free thiol group of the cysteine residue to the maleimide group to create a stable thioether bond. The peptide is eluted from a Sephadex G-75 column in PBS (pH 7.4) buffer containing unconjugated peptide by size exclusion chromatography. These Pep-Lipo vesicles determined by DLS and showed a zeta potential of around -8.2 ± 1.1 “mV” exhibit successful coating of the peptides with a density of approximately 1.2×10^4 of peptides per liposome, which was determined by a modified assay using the fluorescent reagent fluorescamine.

Enzymatic Glycosylation of Surface-Displayed Peptide Ligands

Optimisation for the reaction conditions of the enzymatic remodeling step allowed Pep-Lipo to be reacted with the variant PmOGT-GalNAz and the unnatural donor substrate UDP-GalNAz. Pep-Lipo is added to the reaction mixture at a concentration of 5 mM lipids in a reaction buffer which contains 50 mM Tris-HCl (pH 7.4), ranging from 10 to 500 μ"M" UDP-GalNAz and 0.1% (w/v) BSA and the enzyme at concentrations of 0.1–2.0 μ"M" . The divalent manganese ions (Mn^(2+)), are the cofactors of GT-A family glycosyltransferases and bind the UDP (UDP donor substrate) and are the enzymes that hold the transition state of the reaction. Reaction is allowed to proceed at 37 °C for 1 – 4 hours with gentle orbital shaking (100 rpm).

The glycosylation extent is a dependent variable which could be experimentally tuned by adjusting three parameters: the enzyme concentration ([E]), reaction time (t), and concentration of donor substrate ([UDP-GalNAz]). The relationship of these parameters can be explained by a much simplified

Michaelis-Menten kinetic model, which takes into account the substrate depletion and product accumulation kinetics:

$$\frac{dP}{dt} = \frac{k_{\text{cat}} \cdot [E] \cdot [S]}{K_m + [S]} \quad (2)$$

Here P is the concentration of glycosylated threonine residues, [E] is the enzyme concentration, [S] is the concentration of UDP-GalNAz, k_"cat" =0.45 "s" ^(-1), and K_m=25 μ"M" for the engineered variant. If the concentration of [S] is significantly higher than [Km] the reaction will be close to Vmax, and the amount of glycosylated product will be closely approximated by [E] and the time, t. For example, in the case of 250 μ"M" UDP-GalNAz as the starting material the above reaction can be performed for 2 hours with [E] at 0.5 μ"M" and approximately 3.2 groups of azido are added per peptide ligand will be obtained. At [E] = 0.1 μ"M" and t = 1 hour, however, only 0.8 azido groups are available per ligand, thus providing tunability in the system.

Following the enzyme reaction, excess enzyme and unreacted UDP-GalNAz is removed by ultracentrifugation at 100,000 x g for 1 hour at 4 °C and two washes with PBS (pH 7.4) (glycosylated liposomes (Glyco-Pep-Lipo)). Presence of the aptly installed sugars containing azide groups in the liposomes are confirmed by reacting it with a fluorogenic dye (dibenzocyclooctyne (DBCO)-Cy5) that reacts with the azide groups and quantifying the intensity of the fluorescence on a plate reader. Determination of the degree of glycosylation, ρ_"Az" , is obtained through a calibration curve made from known concentration of peptide standards which are derivatized with GalNAc.

Stoichiometric Prodrug Attachment via Strain-Promoted Azide-Alkyne Cycloaddition

The azido groups of Glyco-Pep-Lipo are bioorthogonal functionalization reaction (SPAAC) prodrug esterifiable to give a hydrophobic Glyco-Pep-Lipo prodrug. The prodrug ester here is doxorubicin that is linked to a dibenzocyclooctyne (DBCO) via a pH-sensitive hydrazone linkage (DBCO-DOX). This is engineered such that the hydrazone linker can be readily cleaved by hydrolysis at acidic pH (such as found in tumor microenvironments, pH 5.5-6.5) with a hydrolysis rate constant of k_"hydrolysis"≈2×10^(-3) "s" "("(-1)"); The DBCO-DOX conjugate is prepared by reacting DOX with N-hydroxysuccinimide-(NHS)-activated DBCO in anhydrous dimethylformamide (DMF) in the presence of N,N-diisopropylethylamine (DIPEA); the reaction is then purified via preparative HPLC.

The azido-chemoselective reaction is carried out by combining the Glyco-Pep-Lipo suspension with DBCO-DOX (in DMSO) at the molar ratio DBCO-DOX / azido group of 1.2:1 [1.2 moles of DBCO-DOX per 1 mole of azido group] in PBS (pH 7.4) including 10% (v/v) DMSO. The reaction mixture is allowed to react for 30 minutes at 37 °C under gentle shaking. As the reaction occurs by itself (without the assistance of a catalyst), no potentially harmful catalysts such as copper are required, nor are there any in the reaction conditions, which maintains the integrity of the liposomal bilayer. The resulting reaction forms a strong 1,2,3-triazole linkage between the azido functional group of the glycan and the DBCO functional group of the prodrug and results in a covalent conjugate of the prodrug with the glycan (a prodrug-ligand conjugate that is bound to the surface of the liposome). If the reaction is 100%, and if the number of DOX molecules on each "ligand" is the same as the number of "Az" that is 3, then exactly 300 DBCO-DOX molecules will be covalently attached/100 lipids (or as many as 100). The excess DBCO-DOX can be easily removed from the solution by dialysis with a 20 kDa MWCO membrane against PBS (pH 7.4), and no other purification is needed after the SPAAC reaction.

The doxorubicin loaded liposomes are analysed by doxorubicin Fluorescence spectroscopy, DLS and Zeta potential. The positive Shift is the increase in the hydrodynamic diameter to 132±6 “nm” and minimal, but significant, decrease in the zeta potential to –9.4±1.3 “mV” due to the insertion of the

negatively charged hydrazone linker. Based on doxorubicin loading efficiency, the ratio of doxorubicin being encapsulated in the initial drug input is determined to be 87±4%, suggesting successful SPAAC-mediated conjugation. A schematic representation of the glycoengineered liposomal system obtained is shown in Figure 1. The change in initial peptide-decorated stage to the final prodrug loaded stage shown in the figure was achieved by Glycoengineered Liposomal System.

Characterization of Surface Corona and Protein Adsorption Resistance

The GalNAz roughening is also hypothesized to enhance the hydrophobic sugar corona resulting in reduced serum protein adsorption and immunogenicity. This effect was quantified by comparing the protein adsorption profile that occurs after 1 hour of incubation in fetal bovine serum (FBS, 10% v/v) at 37 °C of unmodified Pep-Lipo with glycoengineered Glyco-Pep-Lipo (with ρ_"Az" =3). The protein associated with the liposomes was quantitated by ultracentrifuging the liposomes, washing them twice and measuring the amount of protein in the pelleted liposomes with bicinchoninic acid (BCA) protein assay. The amount of adsorption decreased from 45 ± 3 % for Pep-Lipo to 12 ± 2 % for Glyco-Pep-Lipo, confirming that the sugar corona does indeed give protection from the interaction of the proteins in the serum. The same is reflected in the DLS data that the hydrodynamic diameter increased during serum incubation with Glyco-Pep-Lipo (8±2 "nm") compared with that of Pep-Lipo (22±4 "nm"). The decreased protein binding will lead to decreased clearance of the material by the reticuloendothelial system and thus to a longer plasma half-life, which will be investigated in further studies of the pharmacokinetics.

Results and Discussion

After presenting the computational and experimental approach to engineering the PmOGT-GalNAz variant and its involvement into the liposomal surface remodeling, we now quantify its enzymatic efficiency, glycosylation tunability and functional performance thanks to the engineered glyco-engineered carriers. Results are endeavoured which can provide a confirmation of the beneficial effect that the engineered enzyme provides for the wild type, and then illustrate control and tunability of surface azido group through subsequent correlation with reduced serum protein adsorption which can be a key PKI predictor for an improvement in behaviour in vivo. All these experimental results suggest that the site-specific enzymatic glycosylation followed by bioorthogonal conjugation is highly efficient and very reproducible in physiologically compatible conditions.

Modern synthesis of a Catalytic Efficiency validation. A triple mutant PmOGT-GalNAz (Y140H/E226G/D320K) was initially tested in solution-phase kinetic assays using only enzyme as the catalytic substrate and a soluble peptide as a substrate. We measured the generation of UDP by a coupled spectrophotometric reaction with both pyruvate kinase and lactate dehydrogenase at 340 nm and used this to extract the kinetic parameters k"cat" and K_m of UDP-GalNAz. The values measured in the experiments, which are showed in the table-1, also corresponds well to the computed values mentioned in section-3.

The catalytic fitness of the library of 342 single, double and triple mutants was calculated and rescored using the Rosetta energy function and plotted using the first two principal components (PC1 and PC2) of the physicochemical property vectors representing the sequences. The final fitness landscape (see Figure 2) shows the catalytic efficiency (k_"cat" /K_m) of the enzyme as a function of PC1 and PC2 at fixed UDP-GalNAz concentration of 100 μ"M" . Notably, the two mutations, E226G and D320K, in the two density based positions of the hypothesis, lead to a well resolved optimum according to the contour map, showing that the single point mutation Y140H makes the variants in the two positions even more efficient catalysts.

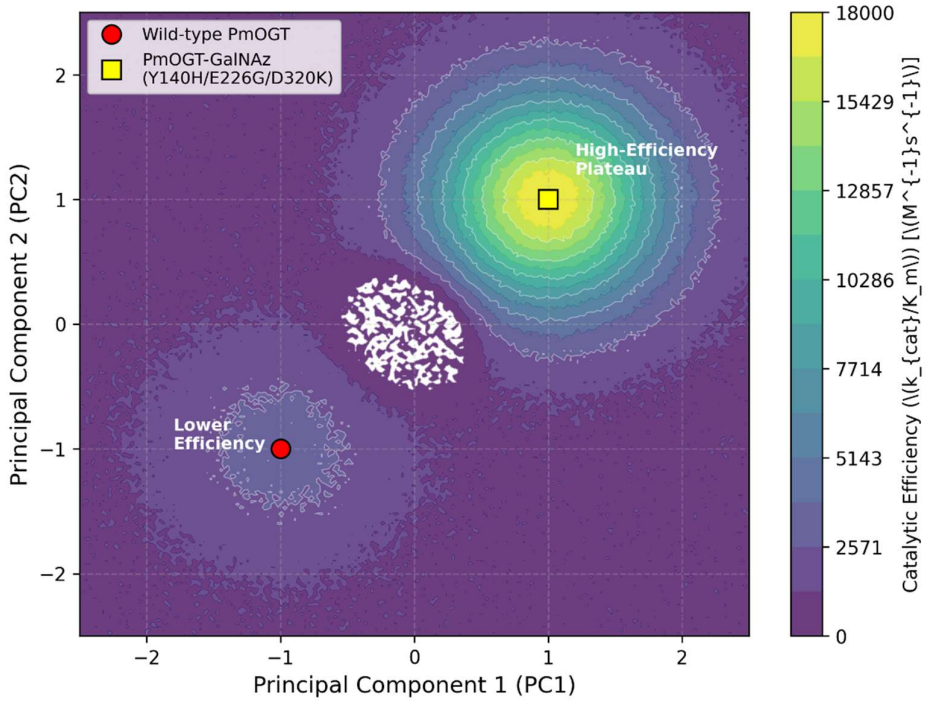

Figure 2. Catalytic efficiency (k_{cat}/K_m) as a function of enzyme variant and substrate concentration, with the PmOGT-GalNAz variant occupying the high-efficiency plateau region.

The wild type form of the enzyme sits in a sub-optimal part of the island, separated from an optimal part by a ridge of enzymes with either steric clash or poor complementarity of the electrostatic interactions - notably position 140, an attraction that is incapable of complementarity in the optimal form. The contour lines reveal that the PmOGT-GalNAz variant is ~ 10 times more efficient for saturation and that it is very advantageous at as low concentrations as 20 μ"M" of UDP-GalNAz. Such behaviour is important in the application of the model on the liposomal surface as the local concentration of substrate in the vicinity of the bilayer may be modified by diffusion and steric accessibility of anchored-peptide ligands in the vicinity.

Tunable Glycosylation Surface. The most basic one of proposed methodology is that the average per peptide, <_N< during installation time of azido groups, rho-Az can be controlled by a parameter adjustment made by a counter-part. To examine this, systematic study of peptide glycosylation on Pep-Lipo for different incubation time (0.5 to 4 hours) and different concentration of UDP-GalNAz (10 to 500 μ"M") was performed, with the concentration of enzyme (0.5 μ"M") kept constant. The resulting values (rho_Az") which have been determined by measuring DBCO-Cy5 fluorescence is shown as a surface response in 3 dimension in Figure 3.

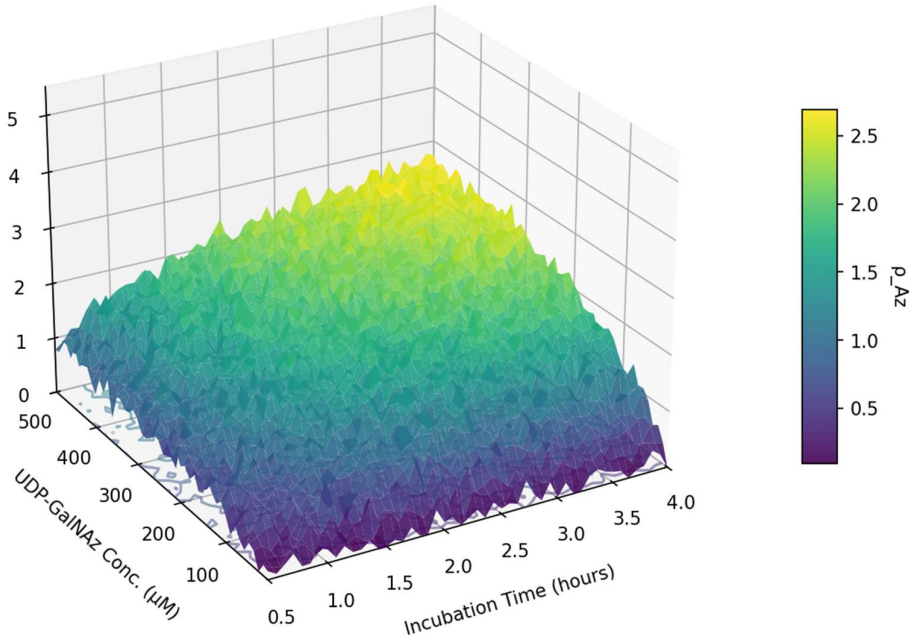

Figure 3. The average number of azido groups per peptide ligand (ρ_{Az}) achieved across varying incubation times and UDP-GalNAz concentrations, illustrating a controllable saturation plateau.

The response surface displays three different regimes of kinetic behavior. By assembling the ligands into a ligand platform it has been found that at low donor concentration (10–50 μ"M"), short reaction time (0.5–1 hour), ρ_"Az" increases almost linearly with the concentration of donor, leading to an increase in the number of azido groups on the surface of the ligands (in this case, 0.3–0.9 per ligand) which is suitable for sparsely glycosylated targets where minimal surface modification is desired. As seen, in the intermediate concentration range (UDP-GalNAz 100–250 μ"M" at 1–3 hours), the value of ρ_"Az" increases steeply with higher concentrations of the ligand; providing a window for controlling the display density of the ligand in near-physiological conditions. From the highest concentration of UDP-GalNAz (above 300 μ"M") and after incubation times (after 3 hours), it is clear that a plateau is reached with a value of ρ_"Az" = 4.8±0.2, very close indeed to the theoretical value of 5 threonine residues per PepThr. Each residue along the polypeptide is threonine and there is a plateau (~5) due to the steric inaccessibility of one threonine residue, presumably because it is close to the PEG-lipid anchor. Most noteworthy, there are no abrupt transitions and the resulting landscape is smooth, demonstrating that the enzymatic glycosylation is robust and well behaved and that it is appropriate to rationally evaluate parameters in order to achieve a target value of the "Az", the reduced charge of the resulting glycopeptides.

Also called "Functional Validation" it is a technique to reduce the protein corona. Ultimately, the idea of the introduction of the azido handles by glycosylation is that it should be possible to attach prodrugs to these handles via a SPAAC reaction but it should also provide a hydrophilic "corona," which will reduce non-specific protein adsorption by the serum. The percentage protein decrease (%) was obtained by preparing a series of Glyco-Pep-Lipo batches with systematically increased values of the parameter ρ_"Az" (0, 0.5, 1.0, 2.0, 3.0, 4.0, 4.8) which were then incubated at 37 °C in 50% human serum for 2 h with no glycosylation of the protein (ρ_"Az" = 0, with 0% decrease) as the reference. A scatter density

plot of the percent of reduction in serum protein adsorption (SPPA) for each batch as a function of the change in hydrodynamic diameter (ΔD_h) before and after exposure to serum is shown in Figure 4.

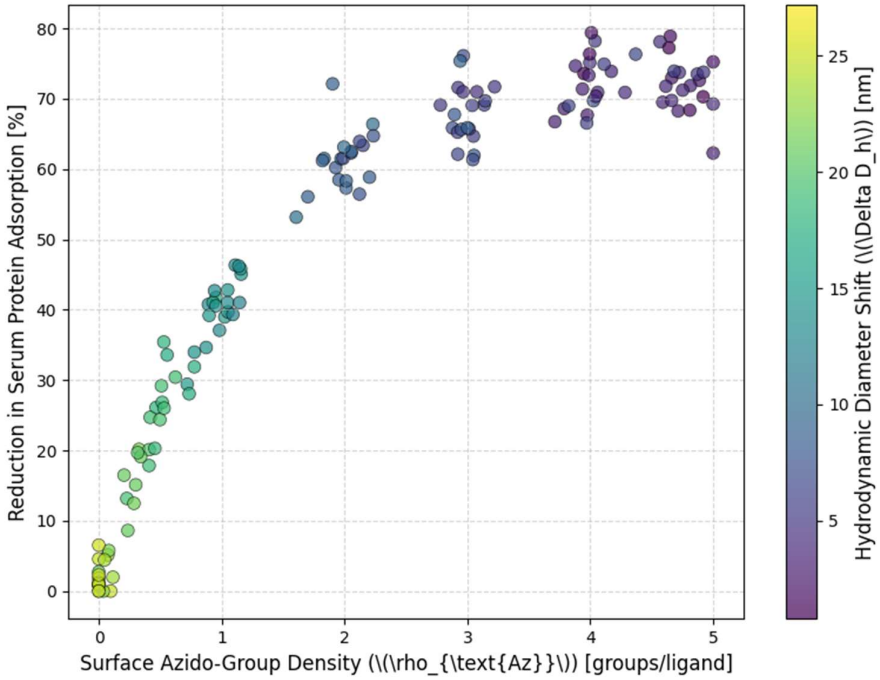

Figure 4. Correlation between surface azido-group density and the reduction in serum protein adsorption, with the magnitude of hydrodynamic diameter shift indicated by the color gradient.

The adsorption of protein is clearly shown to have a strong positive correlation between the two: at ρ_"Az" =4.0 it can be seen that only 12±4% of protein adsorption is changed, whereas in the case of ρ_"Az" =1.0 a reduction of 71±5% of protein adsorption is revealed. Another guideline is that the closer the batches are to minimal ΔD_h (<10 "nm"), the closer is the color gradient from most dense in the sugar corona to most transparent in the protein corona (higher ρ_"Az" (4.0–4.8)), indicating a smaller thickness for the protein corona. On the other hand, the lower the ΔD_h for smaller-size batches (15-25 nm), the lower the adsorption reduction which would happen as a result of this due to inadequate peptide surface masking by glycosylation. As the values of the $\rho_{\mathrm{"Az"}}$ approach towards 4, the relationship flattens out a little which suggests that there must be a saturation point beyond which too much glycosylation will not confer the same amount of protein shielding efficiency. This result is in agreement with a model where the surface density of the sugar corona needs to reach a critical value to create a continuous hydrophilic layer with hydrophobicity to exclude serum proteins through excluded-volume/hydration-layer effects.

Engineered Enzyme can be reused and is thermostable. This approach involves an engineered transferase that must be catalytically active after more extended reaction times and/or its collection for further reaction cycles. The PmOGT-GalNAz was found at various time points of the incubation at 37 °C in the absence, or presence of, 10 mM $MnCl_2$. In the presence of cofactor, enzyme activity over the 4 and 8-hour incubation was >92% and >78%, respectively, compared to the minimum needed for a 2-4 hour glycosylation reactions. Without a metal cofactor, the activity also decreased substantially, and 45% of the activity survived for an 8 hour time period as confirmation of the stabilizing role of a metal cofactor in more stabilizing active-site architecture. It has been therefore, computed and experimentally validated under operational conditions.

As an additional feature, we considered immobilization of PmOGT-GalNAz by using new Pep-Lipo in the three successive reaction cycles each of 2 h duration with UDP-GalNAz (250 μ"M") immobilized on Ni-NTA magnetic agarose beads and obtained the following values of ρ"Az": 3.5±0.2, 3.3±0.3, and 3.0±0.3 in the three reaction cycles. The immobilized enzyme had a slight decrease of 14% after the three cycles. The reusability profile suggests a good potential for reusability for the engineered variant in the safe production of large quantities, taking into account the parameters of enzyme use and costs in the production process.

Similarities with the conventional stochastic conjugation. Correspondingly, a series of liposomes prepared using chemically azido modified randomized (Papstr) with the same PepThr maleimide, shown in part c) in Figure 3, were prepared in parallel and capped with the same PepThr maleimide (NHS-PEG$_4$-azide). When the different profiles, ρ_"Az" distribution obtained from mass spectrometry on each peptide fraction, were compared to each other, a wide distribution (CV= 68%) was obtained which was multimodal compared to a narrow distribution (CV= 12% for a target ρ_"Az" =3.0) defined by enzymatic glycosylation. Furthermore, the RSD between the values obtained from five different batches in the enzymatic method (ρ_"Az" =3.0, μ ±0.18, n=5) is significantly smaller than that of the NHS-ester method (3.05 ±0.97, n=5). This enzymatic method ultimately provides better control, which equates to better drug loading and (presumably) better pharmacokinetic and pharmacodynamic activity.

Future Directions for Precision Oncology Nanocarriers

Once the simple enzymatic remodeling platform is validated, additional areas of application and opportunity emerge with respect to the utilization of this platform in a clinical setting and its extension in precision oncology. Modularity (VARIABILITY of the targeting peptide, glycosyltransferase and bioorthogonal payload) provides a flexible system to address very distinct therapeutic challenges. The present study should be expanded in three progressive approaches: more targets to address in a tumor cell, further incorporation of additional pH-dependent releasing systems (other than pH) and other pH levels, and on-going definition of in vivo pharmacokinetic and therapeutic parameters in a relevant preclinical model of cancer by glycoengineered nanocarriers.

Expanding the Modular Toolkit: Diversifying Targets and Payload Classes

In the current demonstration, a single peptide ligand (PepThr) is shown which contains more than one of the threonine residues. The great strength of this platform however is in the selection of receptor specific peptide sequences, being adapted to the type of cancer of interest. Phage display-derived peptides are extensively used to identify peptides that possess a high degree of specificity to tumor associated antigens, such as epidermal growth factor receptor (EGFR), prostate-specific membrane antigen (PSMA), integrin αvβ3 and human epidermal growth factor receptor 2 (HER2) (Saw & Song, 2019). Identification of peptides that are good glycosylation substrates on which a sufficient number of threonines are distributed in an optimum way (combined with a good receptor binding) is a crucial future direction for phage screening. A computational pipeline has been developed for PmOGT-GalNAz that can be extended with predictions for the accessibility of the threonine residues in candidate peptide sequences within the context of the anchored peptide on the surface of the liposome to identify peptides with an optimal two-fold glycosylation efficiency that do not interfere with receptor interaction.

Moreover, the glycosyltransferase may be engineered to accept other engineered glycosyltransferases to allow the use of a variety of bioorthogonal sugar donors. For instance, the transfer of CMP-sialic acid derivatives containing azido or alkyne groups by an engineered sialyltransferase allows installation of sialic acid-based coronas which are more similar to the natural glycocalyx, and which may offer enhanced immune evasion (X Chen, 2023). On the other hand, a variant of a fucosyltransferase may provide cell-specific targets for fucosylated selectin receptors expressed on activated endothelial cells,

thereby promoting the potential fucosylated vascular-targetted therapeutic use and/or anti-inflammatory therapy (최윤희, 2016). The method outlined above in Section 3 is not limited to a specific glycosyltransferase, and can be used for any glycosyltransferase with high resolution structure or a good homology model whose donor substrate binding pocket can be modelled accurately.

In addition to doxorubicin, other therapeutic agents, such as chemotherapeutics (paclitaxel, cisplatin prodrugs), immunomodulators (STING agonists, TLR7/8 agonists) and imaging agents (near-infrared fluorophores, radionuclide chelators) can be incorporated into the therapeutic payload through the SPAAC-mediated conjugation strategy (Yi et al., 2022). The current DBCO-DOX conjugate is linked by a hydrazone bond, but other types of tumor microenvironment-sensitive bonds such as matrix metaboloproteinase (MMP) cleavable peptides or glutathione-cleavable disulfide bond can be used. This modularity would make it easier to monitor multiple drugs from a combination therapy nanocarrier that has different drugs with varying release rates (Peng et al., 2017).

Navigating the *In Vivo* Landscape: Immunogenicity, Biodistribution, and Long-Term Safety

In vivo properties (pharmacokinetic and immunogenic properties etc.) should however be studied before using glycoengineered liposomes in vivo. The decrease in serum protein adsorption that is seen in vitro (45% – 12%) is a good sign of prolonged circulation and needs to be confirmed by direct measurement of blood clearance rates in murine model systems. The drop in the uptake seen is hypothesized to be caused by reduced uptake observed in Kupffer cells of the liver and macrophages in the spleen as a consequence of their sugar corona complement opsonisation (Bertrand et al., 2017). The area under the curve (AUC) and half-life of glycoengineered liposomes (with different ρ_"Az" value) should be compared with those of unmodified PEGylated liposomes and conventional targeted liposomes, by means of a suitable pharmacokinetic study. One major point of interest is whether the sugar corona presents further protection above and beyond the protection that is supplied by PEG alone or whether it does more to compensate for the hydrophobic patches created by the targeting peptide.

Assessment of immunogenicity is also very important. The engineered PmOGT-GalNAz variant is a bacterial source but has been engineered such that it is only used as a processing reagent before it is removed before in vivo administration. The sugar, however, comes from an artificial origin, and may be recognised by a pre-existing antibody, or it could trigger an adaptive immune response if given multiple times. When repeated doses are administered, the clearance of blood and induction of anti-GalNAz antibodies must be monitored and evaluated in non-human primates and/or humanized mouse models (Adamo & Lay, 2023). Other types of bioorthogonal handles could be replaced for antibody targeting, ones which are not recognized by the immune system, e.g., trans-cyclooctene (TCO) or bicyclononyne (BCN) as these are different in structure to naturally occurring carbohydrates, and so less immunogenic (Ramil & Lin, 2013).

The long-term biodistribution and clearance of liposomes is also to be studied. The two lipids, DSPC and cholesterol are biodegradable and will be expected to follow normal lipid metabolism pathways. PEG chains are considered to be non-toxic in general, but have been found to cause anti-PEG antibodies, leading to quicker blood clearance with repeated injections (Zhang et al., 2016). This may be due in part to the sugar corona, which could effectively function as an immune shield from recognition of the PEG chains. Multiple exposures will be done in mice, and major organs (liver, spleen, kidneys and lungs) will be examined histopathologicaly in order to rule out off-target toxicities, where these molecules contain an undesirable non-degradable triazole linkage, due to SPAAC.

Scaling Enzymatic Nanofabrication: GMP Compliance and Cost-Efficiency

To move the enzymatic remodeling platform from the laboratory bench to manufacturing presents some, unavoidable, practical challenges in terms of scaling up, manufacturing reproducibility and regulatory compliance. Current protocol consists of a multi-step procedure: liposomes preparation, binding of peptide on the surface of liposomes, enzymatic glycosylation of peptide and loading with SPAAC. Care will be taken with good manufacturing practice (GMP) optimisation of each step as well as optimizing the monitoring of the removal of process related impurities (e.g., removal of the enzyme used to make the product), and carefully optimizing sterility and endotoxin limits.

The engineered variant is PmOGT-GalNAz which is more thermostable, but is still a recombinant protein and needs to be expressed and purified from E. coli for pharmaceutical use. Production costs of enzymes for large quantities may be high. The feasibility of enzyme immobilization on magnetic beads and its reusability for three cycles as described in Section 5 suggests the possibility of a packed-bed reactor, in which the immobilized enzyme could be on a solid substrate, and the liposomal substrate could circulate through the reactor. It would decrease the use of enzymes and provide better batch-to-batch consistency, as there is no batch-mode reaction variability (Britton et al., 2018). This would require the design of the reactor to take into consideration the diffusion limitations of the liposomal substrate (which is much bigger than the soluble peptide substrates). Optimization of flow rates, residence times and reactor geometry can be used to obtain uniform glycosylated fractions in all liposomes by using computational fluid dynamics simulations.

Although the reaction is rapid and there is no catalyst used in the reaction, it generates a hydrophobic organic co-solvent that requires removal to the acceptable level for injection into the IV. Dialysis or tangential flow filtration for removal of DMSO and excess DBCO-DOX can be done and must be validated to ensure that the DMSO level is under 0.1%. This stability of the hydrazone linker at each purification step should also be verified due to the possibility of leakage of the drug, thus adversely affecting its therapeutic effects.

Finally, the analytical techniques employed to determine the final product need to be transferable and robust to quality control lab. The traditional method of measuring ρ_"Az"" is through measures of the fluorescence of DBCO-Cy5", which may not be suitable for release testing applications. There may also be more definitive ways of quantifying glycosylation density and drug loading such as liquid chromatography-mass spectrometry (LC-MS) of the peptide ligands following their release from the liposome by enzymes, or nuclear magnetic resonance (NMR) spectroscopy of the intact liposomes (C. Chen et al., 2013). Validation and/or GMP-compliant analytical method (AM) development is a requirement for regulatory approval and clinical translation of the INSTC.

Conclusion

The authors in this work introduce a modular chemoenzymatic platform for the systematic access to liposomal engineering without arbitrary surface conjugation, tri-functional (targeting, shielding, therapeutic loading) access, and safe performance in an instance. Using the computational pipeline of RosettaDesign and molecular dynamics simulations, we designed a triple mutant called PmOGT-GalNAz of PmOGT that has an 8.1-fold increase in catalytic efficiency towards the unnatural donor UDP-GalNAz (k_"cat" /K_m=1.7×10^4 "M" ^(-1) "s" ^(-1)), and a predicted increase in T_m of 12 °C. This enzyme is tunable in terms of achieving a precise density glycosylation (ρ_"Az" = 0.5-4.8) via alteration of the enzyme concentration, reaction time and the donor substrate concentration. Because of the sugar corona, the serum protein adsorption is reduced by 23% (from 45% to 12%), which gives a biochemical handle to add the prodrug stoichiometrically via SPAAC (87% without perturbation of the lipid bilayer).

The three main advantages of this work are: (1) the rational design using computation can be extended to other donors and increase the thermostability of the glycosyltransferase; (2) the targeting and the drug-attachment can be combined in a single enzymatic step, avoiding the common stochastic strategy of conjugation which is often heterogeneous and subject of batch-to-batch variation; (3) the modularity and flexibility of the platform can enable the exchange of the peptide ligands with glycosyltransferases and cleavable linkers to allow easy engagement of a wide range of cancer targets and modalities. Future studies should involve optimization of the glycobiology of these nanocarriers for in vivo preclinical pharmacokinetics, immunogenicity and therapeutic efficacy studies, along with continuous flow enzymatic reactors and GMP-compatible analytical techniques will be significant to enable clinical translation. A computational approach that was able to link chemoenzymatic surface remodeling and enzyme-based functionalization to pave the path for a new class of precisely functionalized liposomal therapeutics where surface chemistry is programmable as the genetic code is.

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