Summary
Development of new methods for linking sugars to peptides or proteins is an active area of research because natural glycopeptides or neoglycoconjugates play important roles in biology and medicine and are indispensable tools for probing several biological processes. However, glycoconjugate synthesis remains a formidable task as synthetic protocols are quite demanding and involve multiple reaction steps with requirements of extensive protection of reactive functionalities. Given the current ease with which peptides are assembled by solid phase methodology and proteins obtained from expression systems, the availability of enzymes capable of covalently linking a pre-synthesized sugar and a polypeptide would greatly facilitate the synthesis of glycoconjugates.

The membrane-anchored sortase enzymes present in gram positive bacteria are a group of cysteine transpeptidases that catalyze the anchoring of several cell surface proteins to the cell wall peptidoglycan. The prototypical sortase, sortase A of *Staphylococcus aureus*, recognizes a LPXTG like sequence motif located near the C-terminus of the target proteins, cleaves at Thr-Gly peptide bond, and catalyzes the formation of a new peptide bond between threonyl carboxyl and amino group of the pentaglycine cross-bridges of peptidoglycan. The transpeptidation reaction proceeds in two steps without the aid of any extraneous molecule; the active site cysteine residue first attacks the target LPXTG substrate forming an acyl-enzyme intermediate which in the second step is resolved by the nucleophilic attack of the amino group of the terminal glycine residue of the peptidoglycan. In the absence of a suitable amino nucleophile, the LPXTG peptide substrate is slowly hydrolyzed.

Sortase mediated transpeptidation reaction involving LPXTG and aminoglycine containing polypeptides proceeds smoothly *in vitro* and the feasibility of using sortase in semi-synthetic strategies has been tested in some cases. It has been used to synthesize peptide-peptide, protein-peptide and protein nucleic acid–peptide conjugates that would have been difficult to assemble by purely chemical or genetic means. Interestingly, ligation of LPXTG substrates can occur even with polypeptides containing a single glycine residue at the amino terminus. This observation of relaxed specificity for the amine nucleophile prompted further exploration of sortase-mediated ligation of polypeptides to amino sugars with a view to develop an enzymatic approach for synthesis of glycoconjugates with potential bioactivity.
SrtA<sub>ASS</sub> sequence corresponding to amino acids 60-204 was amplified from the <i>Staphylococcus aureus Mu50</i> genomic DNA and cloned into pET23b vector. Purification of protein was carried out as per standard Ni-NTA procedure. The identity of recombinant sortase was established by Electrospray (ES) mass spectrometry (MS) and Edman degradation. 6-aminohexoses were considered as potential sugar substrates with the idea that the -CH<sub>2</sub>-NH<sub>2</sub> moiety present in these sugars might mimic some elements of the glycine structure. Accordingly, the potential of sortase to ligate 6-amino-6-deoxyglucose and 6-amino-6-deoxymannose to the YALPETGK peptide substrate was tested. HPLC assays followed by MALDI-TOF or ESMS analysis revealed the formation of the respective YALPET-sugar adducts suggesting that the above amino sugars acted as nucleophiles in the transamidation reaction. To further probe the specificity requirements as well as to see if 6-aminohexoses can serve as recognition tags for peptide-sugar ligations, the ability of sortase to ligate peptides the aminoglycoside class of therapeutically important antibiotics was examined. Sortase mediated ligation of YALPETGK to aminoglycoside proceeded as expected. Analyses of the reaction products by RP-HPLC followed by MALDI-TOF revealed the formation of specific conjugates between antibiotics and YALPETGK in the yields varying from 35-70% for the kanamycin class, and 18-30% for the ribostamycin class of antibiotics. ESMS of the respective conjugates produced fragmentations that unambiguously showed occurrence of peptide ligation exclusively at a single 6-amino site in ring A of the kanamycins, tobramycin and ribostamycin or ring D of paromomycin and neomycin. Thus, conjugation of peptide substrates was limited to the 6-amino site in the antibiotics despite the presence of a plethora of amino groups, indicating rather strict specificity and selectivity for the sugar amino groups by sortase.

Transpeptidation of LPXTG peptide substrates to aminoglycosides amikacin and butirosinA that belong to the kanamycin and ribostamycin class respectively but contain an additional side chain with a terminal amino group was also investigated. Both these antibiotics produced peptide-aminoglycoside adduct at the amino group present in the butyryl side chain in addition to that at the 6-amino site. Interestingly, the butyryl side chains, 4-amino-2-deoxy butyric acid and 4-amino-3-hydroxy butyric acid, independently acted as substrates in the sortase catalyzed transpeptidation reaction. Likewise, 6-amino hexanoic acid, 7-amino heptanoic acid,
6-aminohexanol, spermine, cadaverine and agmatine also formed adducts with LPXTG peptides.

The feasibility of using sortase for site-specific conjugation of sugars to proteins was also tested. For this a protein (Mrp, NP_372281) from *Staphylococcus aureus* nested with a LPNTG sequence motif in its carboxy terminal region was expressed. Incubation of Mrp with sortase in the presence of tobramycin produced specific conjugates (Mrp<sub>1,170</sub>-Tobramycin). Similar conjugates were also obtained with other aminoglycoside antibiotics.

The conjugation of biologically relevant peptides to aminoglycoside antibiotics was also explored. Towards this, peptides derived from Tat and Rev proteins of HIV were considered because these proteins play important roles in viral replication through their interactions with structured viral RNA targets; TAR in case of Tat and RRE in case of Rev. Aminoglycosides and peptides derived from Tat and Rev have been independently shown to interfere with Tat-TAR and Rev-RRE interactions leading to inhibition of viral replication. Using sortase-catalyzed transpeptidation, conjugates of neomycin, tobramycin and ribostamycin with Rev and Tat sequences appended with an LPETG motif were prepared. The ability of these conjugates to bind to their respective RNA was evaluated by electrophoretic mobility gel shift assays. The gel shift assays for the Neomycin/tobramycin-Rev conjugates yielded a RRE binding affinity that was about 10-12 fold higher as compared with the native Rev peptide. In contrast, the binding affinities of the Tat-aminoglycoside conjugates were found to be more or less similar to the Tat peptide. The overall results suggested the utility of using sortase-catalyzed transpeptidation for the generation of tight RNA binders with potential bioactivity.

In summary, the work presented in this thesis demonstrates that *Staphylococcus aureus* sortase can transfer peptide substrates to oligosaccharides appended with a 6-deoxy-6-aminohexose moiety in a selective manner as that of an oligoglycine sequence. This reaction provides a simple and straightforward method for covalent ligation of a prefabricated sugar containing a 6-aminohexose tag to synthetic peptides and expressed proteins encoded with a C-terminal LPXTG sortase recognition sequence. The reaction can be exploited for the facile assembly of aminoglycoside antibiotic-peptide conjugates with improved RNA binding properties. In addition, the results show that sortase can transfer LPXTG containing
peptides/proteins to a plethora of substrates such as α-amino hydroxy acids, long chain aliphatic amines and aminoalcohols/acids. Sortase appears to be a highly promiscuous enzyme endowed with tremendous synthetic capability.