Heparin, a highly sulfated glycosaminoglycan (GAG), is used extensively as an anticoagulant. It consists of repeating disaccharide units, containing iduronic acid (or glucuronic acid) and glucosamine, exhibiting variable degrees of sulfation. Heparin, and its analogues, are used during surgery and dialysis, and are often used to coat indwelling catheters and other devices where the vascular system is exposed. Administered parenterally, often continuously due to its short half-life, over 0.5 billion doses are required per year. The annual sales of pharmaceutical heparin are over $3 billion and it is prepared in 100 metric ton amounts annually. Currently obtained from mucosal tissue of meat animals, mainly porcine intestine, and to a lesser extent bovine lung, its early stage production is poorly controlled, due to the source of the material. This problem came into sharp focus in 2008 when the presence of contaminating over-sulfated chondroitin sulfate in heparin, sourced from pigs, resulted in 81 deaths in the USA. This, coupled with the fact that only two doses are obtained per animal means that the demand for an alternative and more controlled sources of heparin is high.

To this end the Glycoengineering subgroup consists of two sections: Bioengineering and Metabolic Engineering. The bioengineering section involves the use of microbial, mainly bacterial, processes to generate and modify GAGs, using a combination of fermentation and chemo-enzymatic modification. The metabolic engineering section involves the manipulation of the natural metabolic processes of mammalian cells to generate and modify GAGs, intracellularly.

Bioengineered Heparin Project

A biosynthetic pathway was designed that began with bacterial synthesis of the backbone structure, N-acetyl heparosan, with the aim of producing a bioengineered heparin product. N-Acetyl heparosan is synthesized by E. coli K5 as a capsular polysaccharide and is shed into the growth media.
Once extracted and purified, the heparosan polysaccharide undergoes a series of *in vitro* chemoenzymatic modifications to make heparin, with the initial steps (N-deactylation and N-sulfonation) involving chemical modification and the final steps (epimerization of glucuronic acid to iduronic acid, 2-O-, 6-O-, and 3-O-sulfation, involving enzymatic modification. Since heparin production would be reliant on microorganisms, instead of animals, scale up could be achieved by increasing fermentation volume of the heparosan producer, *E. coli* K5, and the recombinant *E. coli* strains used to produce the heparosan modifying enzymes. The process has been completed on a small scale and is being optimized in order to pursue heparin synthesis on a larger scale that could eventually be scaled up to produce bioengineered heparin at an industrial scale.
In a related project, in collaboration with the Koffas lab, we have recently sequenced the genome of *Escherichia coli* K5 and have constructed a draft genome-scale metabolic reconstruction to improve the endogenous K5 capsular polysaccharide (heparosan) production capacity of this *E. coli* strain using stoichiometric-based modeling. A multilevel optimization algorithm capable of predicting combinations of genetic manipulations for increased production of target metabolites has been constructed by building upon the success of Cipher of Evolutionary Design (CiED), a genetic algorithm developed in the Koffas lab for predicting gene knockouts.
Application of the MATLAB-based algorithm to the newly constructed metabolic model allows prediction of sets of gene overexpressions and deletions consistent with the desired cellular phenotype, high heparosan production levels. Computational predictions for improving availability of heparosan precursors are consistent with previous experimental results and are being evaluated in the lab.

We are also utilizing traditional metabolic engineering approaches aimed at increasing intracellular supply of heparosan precursors, such as overexpression of the capsular polysaccharide gene cluster, overexpression of enzymes in the metabolic pathway, and elimination of nonessential competing pathways by targeted gene deletion.

Mutant *E. coli* K5 strains possessing a combination of rational and predicted genetic manipulations have the potential to achieve significantly improved heparosan titers, making *E. coli* a safe and viable heparosan production platform as a source for bioengineered heparin.

**Metabolic Engineering Project**

Heparin, a natural product of mast cells, is stored in mast cell granules and is released with histamine on type-1 allergic response leading to mast cell degranulation. Metabolic engineering offers a potential approach to prepare heparin by fermentation. The metabolic engineering of bacterial cells is relatively straightforward since most of the biosynthetic pathways are relatively simple and well known, but while bacteria are both easy and inexpensive to engineer and to grow, they are not compartmentalized, lack a Golgi and thus are incapable of making all but the
simplest glycoproteins. Yeasts are eukaryotes, and have a Golgi, but do not make GAGs and are missing the required ability to sulfate polysaccharides. Mast cells cannot be grown in culture and even when converted to mastocytoma cell lines grow very poorly and thus are not good candidates for metabolic engineering.

Chinese hamster ovary (CHO) cells are an ideal candidate for the production of heparin. They already have the metabolic apparatus required to make the related GAG, heparan sulfate, and are used in the commercial production of glycoprotein drugs and thus are the target of our metabolic engineering studies. We bioengineered CHO cell lines, which use the heparan sulfate biosynthetic pathway to produce heparin, by transfecting them with enzymes required for anticoagulant heparin production. Anticoagulant activity of the final product was only modestly increased, which led us to study the enzymes of the heparin biosynthetic pathway in more detail with the aim of improving upon our system.

We used bioengineered mastocytoma cells, which produce anticoagulant heparin, to study the localization and activity of heparin biosynthetic enzymes. Data obtained from these studies led us to bioengineer CHO cell lines with optimal localization of the enzymes.

Currently we are working on the development of assays to assess the activity of the bioengineered enzymes. We are also comparatively profiling HS/HP biosynthetic enzymes, their effect on HS/HP composition and HS/HP biological activity in CHO stable clones and other cell-lines, including the mastocytoma cell line.
Bioengineering of Heparin in CHO cells

**Step 1**
Establish expression level of enzymes, involved in HP/HS biosynthesis
- NDST 2 - C5Epi - 2OST - 6OSTs - 3OSTs

**Step 2**
Transfection/ Overexpression of enzymes that CHO cells lack

**Step 3**
Heparin Production