Enzyme immobilization for the controlled release of a bioactive molecule from hydrogels

Casey Donahoe, Megan M. Kaneda, Shannon K. Alford, Bradley K. Wacker, Evan A. Scott, Donald L. Elbert
Department of Biomedical Engineering, Washington University in St. Louis, St. Louis MO 63130

Statement of Purpose: Drug delivery from engineered scaffolds may greatly enhance the integration of devices with native tissues. However, tremendous challenges remain for drug delivery from hydrogels, including first-order release profiles and volume limitations. For example, hydrogel coatings for endovascular stents might reduce the incidence of thrombosis, however, the amount of drug required for the coating (e.g. 250 mg) makes the use of water-swollen materials impractical. Production of the active molecules *in situ,* via immobilization of active enzyme, will lead to near zero-order release of product, if the concentration of the drug precursor is above the K_M of the enzyme. While this strategy is not practical for many drug systems, an example exists with the bioactive lipid sphingosine 1-phosphate (S1P). S1P is produced from sphingosine by the enzyme sphingosine kinase (SphK).[1] Extracellular S1P interacts with G-protein coupled receptors on endothelial cells to promote migration, while inhibiting migration of smooth muscle cells. S1P is also a complete angiogenic factor.[2] Therefore, we hypothesized that immobilization of SphK in tissue engineering scaffolds will promote angiogenesis and immobilization on stents and vascular grafts will enhance endothelialization.

Methods: *Cloning of SphK* – HUVEC (Clonetics, Walkersville, MD) were lysed and homogenized using a Qiashredder column (Qiagen, Valencia, CA) and mRNA was collected using the RNeasy kit (Qiagen). RT-PCR was performed with 50 ng HUVEC mRNA with a Qiagen One Step RT-PCR kit and primers 5'-GACTG AATTCATGGATCCAGCGGGCGGCCCC and 5'-GAC TCTCGAGTCATAAGGGCTCTTCTGGCGGTGGCAC GT. RT-PCR products were verified using agarose gel electrophoresis and purified using the Nucleotide Removal kit (Qiagen). Purified RT-PCR products were cloned into the pDrive cloning vector according to the protocols of the AU Cloning kit (Qiagen). Insertion of product was verified by PCR and agarose gel electrophoresis. The PCR insert with the correct sequence was transferred from the cloning vector to the expression vector, pGEX-6P-1, using the restriction enzymes EcoRI and XhoI. The plasmid with the correct sequence was transfected into BL21 E. coli and the cells were grown to OD 0.6 in LB medium. Protein expression was induced with 1 mM IPTG. After 3 h, the cells were harvested by centrifugation for 20 min at 6000 *g*, 4°C. The cells were lysed with lysozyme and sonication. The lysate was centrifuged at 50,000 *g* for 45 min at 4°C and the supernatant was filtered. The protein was purified using GSTrap FF columns. Activity of the protein (10 µg/mL) is assessed by incubation with 50 µM D-erythro-sphingosine in 0.1% BSA in PBS for 60 min, followed by dilution 1:1 with methanol and acidification to pH 1 with HCl. Lipids were extracted into chloroform, which was then dried. Lipids were analyzed by thin layer chromatography.

Sphingosine kinase gels – Hydrogels were produced by incubating 4 mg of PEG-octavinylsulfone with 50 µL of 25 µM purified, GST-tagged sphingosine kinase for 1 h, with reaction by Michael addition. Then, 6 mg of albumin was added to crosslink the PEG at 37°C for 24 h. Each gel was washed with PBS for 24 h. Reaction mixtures contained 500 µM sphingosine and 1 mM ATP.

Results/Discussion: Sphingosine is present in blood at concentrations of 170 nM.[3] ATP is present in blood at 170 nM and binds strongly to albumin.[4] We produced PEG/albumin/SphK hydrogels and demonstrated by thin layer chromatography that the sphingosine kinase was active after incorporation into the hydrogel. Complete conversion of the sphingosine to S1P was observed within 48 h.

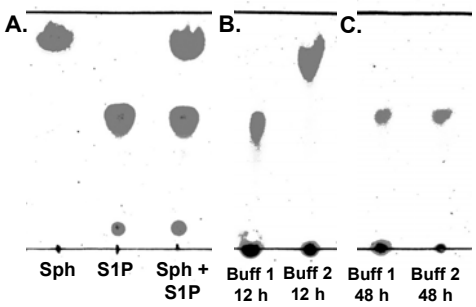

Figure1 –Reaction mixtures containing 500 µM sphingosine and 1 mM ATP in: (Buffer 1) 100 µL Buffer A: 400 µL PBS, or (Buffer 2) PBS with 0.02% BSA. At different times, the supernatants were removed and gels were washed for 24 h with PBS containing 0.4% BSA. Lipids were extracted from these solutions using chloroform/methanol. (A) S1P and sphingosine controls. (B) Reaction after 12 h. (C) Reaction after 48 h. Buffer A is 20 mM Tris-HCl, pH 7.4, 10% glycerol, 0.05% TritonX-100, 1 mM dithiothreitol.

Conclusions: The precursor to sphingosine 1-phosphate is present in blood, providing an opportunity for an alternative drug delivery strategy for S1P from materials. We demonstrated that the enzyme was active in the hydrogel and completely converted sphingosine to S1P.

References:

[1] Kohama, T. et al., *J Biol Chem*, 1998. 273: 23722-8.
[2] English, D., et al., *FASEB J*, 2000. 14: 2255-65.
[3] Deutschman, D.H., *Am. Heart J.*, 2003, 146, 62-68
[4] Douillet, C.D., *Luminescence*, 2005, 20, 435-441