Role of Nitric Oxide-Releasing Glycosaminoglycans on In Vitro Wound Healing

Sara E. Maloney[a] and Mark H. Schoenfisch[a,b]
[a]Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599
[b]Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Statement of Purpose. In the United States, as many as 6.5 million people suffer from chronic wounds.[1] Unlike acute wounds, which heal without excessive intervention, chronic wounds display prolonged inflammation that can remain indefinitely, especially when potentiated by persistent infection. The high risk of amputation and mortality associated with chronic wounds necessitates new, efficacious wound healing therapeutics. Nitric oxide (NO) represents a dual-action alternative to conventional therapies due to its roles in the inflammatory response and its potent antimicrobial action.[2] As a result of NO's short biological lifetime and cumbersome treatment requirements, the use of macromolecular NO donors can facilitate localized delivery. The endogenous glycosaminoglycans hyaluronic acid (HA) and chondroitin sulfate (CS) are integral to endogenous wound healing. Molecular weight-dependent signaling by HA leads to inflammatory cell recruitment, collagen deposition, and keratinocyte proliferation.[3] CS is upregulated in granulation tissue and is involved with fibroblast proliferation and adhesion.[4] These functions make HA and CS attractive NO donor scaffolds for wound healing.

Methods. Carbodiimide chemistry was used to modify the carboxylic acid groups of hyaluronic acid (6 kDa, 50 kDa, and 90 kDa), chondroitin-4-sulfate (CS-A; 30 kDa), and chondroitin-6-sulate (CS-C; 20 kDa) with a series of alkylamines. Amine-modified HA and CS derivatives were reacted with high pressure gaseous NO under alkaline conditions to form *N*-diazeniumdiolate NO donors on the polysaccharide backbones. Amine-modification was assessed using ^{13}C NMR, FTIR, and elemental (CHN) analysis. Real-time NO-release properties were evaluated in simulated wound fluid (SWF) using a chemiluminescent nitric oxide analyzer. Antibacterial efficacy was evaluated against common wound pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and antibiofilm efficacy was tested using *P. aeruginosa* biofilms. In vitro cytotoxicity of the HA and CS derivatives was assessed with human epidermal keratinocytes (HEK) and human dermal fibroblasts (HDF) as representative cell types for a wound environment. Multiple in vitro assays, including cell migration, cell adhesion, and cell proliferation assays, were performed to assess wound healing characteristics of HA and CS as a function of polysaccharide identity, molecular weight (HA), alkylamine modification, and NO donor loading.

Results. Nitric oxide-releasing HA and CS-C derivatives modified with bis(3-aminopropyl)amine (DPTA) stored 0.3-0.9 μmol NO mg^{-1} and exhibited sustained release (half-lives of 20-60 min) in SWF. While CS-C-DPTA/NO had large NO payloads (~0.9 μmol mg^{-1}), CS-A modified under the same conditions stored much less NO (<0.1 μmol mg^{-1}), likely a result of sulfation patterns and/or monomer ratio. Broad-spectrum bactericidal activity (>3-log reduction in bacterial viability) was achieved with both HA and CS-C derivatives. The NO-releasing DPTA-modified polysaccharides eradicated (>5-log reduction in bacterial viability) *P. aeruginosa* biofilms (Figure 1), with HA6-DPTA/NO having the most potent antibiofilm activity and CS-C-DPTA/NO the least. In vitro cytotoxicity against HEK and HDF revealed IC_{50} values of 1-8 mg mL^{-1} for NO-releasing derivatives and 3 to >32 mg mL^{-1} for non-NO-releasing derivatives, influenced by the NO loading and alkylamine structure. In vitro cell migration assays using HEK found that 100 ng mL^{-1} treatments of HA6-DPTA/NO and CS-C-DPTA/NO facilitated faster migration than their non-NO-releasing counterparts as well as HA50-DPTA/NO and HA90-DPTA/NO. High concentrations of HA6-DPTA/NO (i.e., 1 mg mL^{-1}) hindered HEK migration.

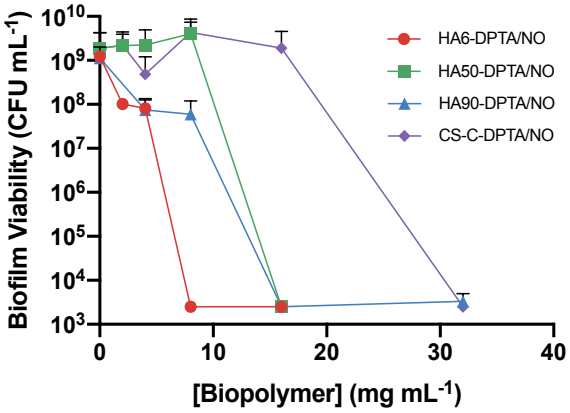

Figure 1. Antibiofilm action of NO-releasing HA and CS against *P. aeruginosa* biofilms

Conclusions. Nitric oxide-releasing HA and CS derivatives represent promising dual-action wound therapeutics due to their ability to both eradicate biofilms and promote cellular migration. The HA derivatives were found to be stronger antibiofilm agents than CS derivatives, which can be attributed to CS's sulfate groups hindering association with the negatively charged bacterial membrane. The 6 kDa HA and CS-C derivatives were most effective at promoting HEK migration, demonstrating that NO-releasing HA6 and CS-C may promote in vivo wound healing. Additional in vitro assays are underway to further evaluate the NO-releasing glycosaminoglycans.

Disclosure Statement. Mark H. Schoenfisch is a co-founder of and maintains financial interest in KnowBio, LLC., a company commercializing NO-releasing macromolecular vehicles.

References.

(1) Olsson M. *Wound Rep. Reg.* 2019; 27; 114-125.
(2) Carpenter AW. *Chem. Soc. Rev.* 2012; 41; 3741-3752.
(3) Graça MF. *Carbohydr. Polym.* 2020; 241; 116364.
(4) Zou XH. *J. Dent. Res.* 2004; 83; 880-885.