PEG-BPEI Loaded Sponges Neutralize Pathogen Virulence

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METHODS OF TREATING BACTERIAL INFECTIONS WITH PENAM β-LACTAM ANTIBIOTICS AND BRANCHED POLY(ETHYLENIMINE) US patent US10953040B2

ANTIBIOTIC AND ANTI-INFLAMMATORY COMPOSITIONS AND METHODS OF USE
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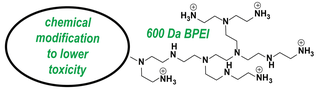
INTRODUCTION

The development of chronic wounds is a significant problem in medical centers. In rural areas, the problem is worse because of limited access to medical resources. The Public Policy Committee of the National Pressure Injury Advisory Panel calls for "dedicated clinical course correction in acute and postacute care" before the prevalence of pressure injuries becomes a public health crisis. Fortunately, the National Pressure Injury Advisory Panel also notes that "…Most PIs are preventable." In response, we propose a novel strategy for the treatment of chronic wounds, including the mitigation of progression to advanced stages, with PEGylated branched polyethyleneimines (PEG-BPEIs) in collagen sponges.

Chronic wounds share a common clinical indication – excessive inflammation – that is caused, in part, by bacterial pathogens and tissue damage that trigger immunological alarm signals. However, the dysregulation of wound healing arises from damaged tissue, infectious agents, biofilms, and toxins. Loading PEG-BPEI into collagen sponges represents a novel wound healing and infection prevention strategy that is easy to administer and will prevent bacterial colonization, biofilm formation, and sepsis with extended activity. These collagen sponges can easily be applied to wounds, even by the patient themselves, reducing the need to visit a medical facility for treatment.



PEGylated 600 Da BPEI (PEG-BPEI)

DIFFERENTIATION FACTORS

Our technology addresses (1) antimicrobial resistance (AMR) against standard-of-care antibiotics; (2) biofilms that stifle host defense and antimicrobial activity; and (3) pathogen-associated molecular pattern molecules (PAMPs) that stimulate the inflammasome. The mode of action is electrostatic binding to lipopolysaccharides, teichoic acids, peptidoglycan, and biofilm extracellular polymeric substances.

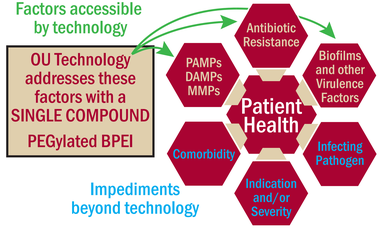
ADVANTAGES:

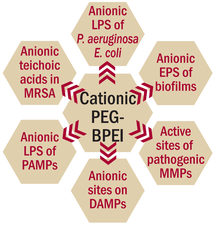
- PEG-BPEI is NOT a peptide; thus, it resists proteolysis
- broad-spectrum utility against AMR from Gram-positive and Gram-negative pathogens
- broad-spectrum utility against biofilms from Gram-positive and Gram-negative pathogens
- broad-spectrum utility against PAMPs from Gram-positive and Gram-negative pathogens

PROPERTIES:

- Easy and straightforward one-step synthesis
- Hydrophilic properties and very-high water solubility to enable easy drug delivery
- There are minimal protein binding effects that would prevent activity; demonstrated with antibiotic potentiation assays using fetal bovine serum (FBS).
- Minimal *in vitro* cytotoxicity
- *In vivo* toxicity issues are paramount, which are mitigated with chemical design

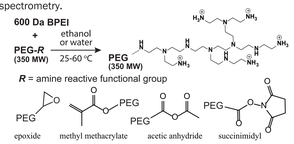
MULTI-FACETED THERAPEUTIC STRATEGY FOR ANIONIC TARGETS





EASY 1-STEP SYNTHESIS

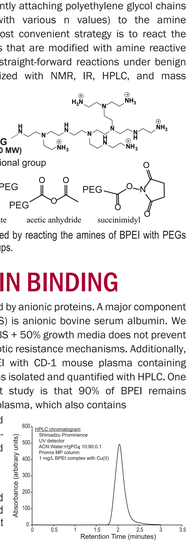
Graft Copolymers of 600 Da BPEI with PEG-derivatives can be readily obtained by covalently attaching polyethylene glycol chains (PEGs, $CH_3[OCH_2CH_2]_n$ with various n values) to the amine nitrogens of BPEI. The most convenient strategy is to react the amines of BPEI with PEGs that are modified with amine reactive functional groups. These straight-forward reactions under benign conditions are characterized with NMR, IR, HPLC, and mass spectrometry.



PEGylated 600 Da BPEI formed by reacting the amines of BPEI with PEGs containing amine-reactive groups.

LOW PROTEIN BINDING

BPEI activity is not inhibited by anionic proteins. A major component of fetal bovine serum (FBS) is anionic bovine serum albumin. We demonstrated that 50% FBS + 50% growth media does not prevent BPEI from disabling antibiotic resistance mechanisms. Additionally, after mixing 600 Da BPEI with CD-1 mouse plasma containing albumin, unbound BPEI was isolated and quantified with HPLC. One conclusion from the pilot study is that 90% of BPEI remains unbound in mouse blood plasma, which also contains anionic sulfates and carboxylates on glycos-aminoglycans heparin and dermatan sulfate.



600 Da BPEI can be measured in blood plasms with HPLC and using a Cu(II) the complex at 275 nm.

TECHNOLOGY APPLICATIONS

PEG-BPEI is a novel agent that simultaneously, and independently, neutralizes PAMPs, antibiotic resistance mechanisms, and biofilms using electrostatic forces. This versatility of PEG-BPEI suggests that it may be a useful therapeutic against wounds, pressure ulcers, and burns by simultaneously providing anti-inflammation and infection prevention with a new innovative product and decolonizing agent. Reducing the activation of toll-like and node-like receptors will suppress the expression of pro-inflammatory cytokines and harmful MMPs. This will speed wound healing while also slowing the emergence and spread of antibiotic resistance genes and antibiotic-resistant pathogens.

Skin and Soft Tissue Infections

Burns	Surgical Wounds
Acute wounds	Skin Diseases
Chronic wounds	Animal Health

MINIMAL CYTOTOXICITY

Off-target effects were evaluated with human cells. We examined the cytotoxicity by measuring the IC_{50} value, which is the amount of compound required to inhibit proliferation of half of the cells. Higher IC_{50} values are indicative of less toxic drugs. Here, IC_{50} values indicate that PEG1000-BPEI has the lowest cytotoxicity, followed by PEG350-BPEI and 600 Da BPEI.

IC_{50} against human THP-1 macrophages

- 170 μM for 600 BPEI
- 274 μM for PEG350-BPEI
- 951 μM for PEG1000-BPEI

IC_{50} against human HeLa cells

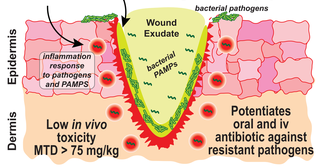
- 396 μM for 600 BPEI
- 794 μM for PEG350-BPEI
- 1350 μM for PEG1000-BPEI

acute toxicity > 75 mg/kg

MARKET SUPERIORITY

OU Technology Provides Market Superiority

Topical non-peptide agent that is easily formulated
Disrupt biofilms
Prevents PAMPs from activating inflammatory cytokines



Ideal Treatment	Reality
☐ kill bacteria	☒ AMR prevents killing
☐ disrupt biofilms	☒ limited anti-biofilm agents
☐ alleviate inflammation	☒ LPS production continues

Ideal Treatment	PEG-BPEI
☐ kill bacteria	☑ disables AMR mechanisms
☐ disrupt biofilms	☑ disperses biofilms
☐ alleviate inflammation	☑ neutralizes LPS endotoxins

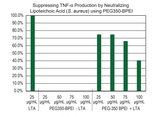
PRE-CLINICAL STUDIES

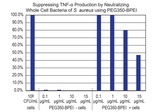
PEGylating one of the primary amines on 600 Da BPEI lowers single-dose acute toxicity. Toxicity data were collected by TransPharm Preclinical Solutions Inc., a contract research lab. The acute toxicity LD0, or maximum tolerable dose (MTD), was evaluated over 3 days using female ICR mice with daily subcutaneous q24h dosing. For 600 Da BPEI, the MTD is 25 mg/kg. A single 350 MW PEG on BPEI (PEG350-BPEI) increases MTD to 75 mg/kg. These data show that subcutaneous PEG-BPEI, for instance applied to a wound or burn with exposed tissue layers, has lower acute toxicity and is safer to use than 600 Da BPEI.

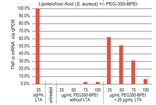
INFLAMMATORY CYTOKINES

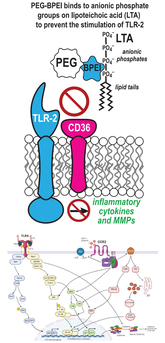
Inflammation occurs in response to alarm signals present in the microenvironment. Pathogens release signaling molecules known as Pathogen-Associated Molecular Pattern molecules (PAMPs). For instance, *S. aureus* is a leading cause of wound infections and invades the host, causes disease, avoids drug activity, and evades host defenses through the release of PAMPs that stimulate the release of pro-inflammatory cytokines.

We have used Enzyme-linked immunosorbent assay (ELISA) to measure the amount of TNF-α cytokines released from THP-1 macrophages. LTA and whole cell bacteria activate the release of TNF-α cytokine. The amount of TNF-α is reduced when the antigens are pre-mixed with PEG-BPEI. Activation of toll-like-receptors (TLR) induces a signaling cascade. Data show that PEG-BPEI blocks TLR activation by lowering mRNA levels, measured with qPCR. We can also block the production of other pro-inflammatory cytokines with PEG-BPEI (data not shown).

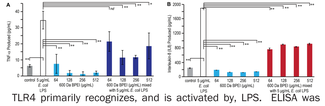








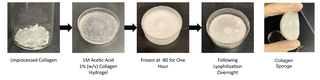
Inhibitory effect of PEG-BPEI on TNF-α cytokine production in cell culture supernatant of *S. aureus* LTA-stimulated THP-1 macrophages using ELISA. THP-1 cells were incubated with LTA, PEG-BPEI, and combo for 4 h; or with heat killed SA, PEG-BPEI, and combo for 6 h. The mRNA expression level of TNF-α was detected by qPCR. PEG-BPEI, and untreated cells were used as negative control. Data are shown as mean ± SD (n = 3).



TLR4 primarily recognizes, and is activated by, LPS. ELISA was used to determine the amount of TNF-α and IL-8 released from HaCaT keratinocytes after *E. coli* O111:B4 LPS stimulation. Mixing 600 Da BPEI with LPS (5 μg/mL) results in a reduction in TNF-α production and a reduction in IL-8 production.

COLLAGEN SPONGES

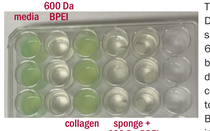
Collagen sponges imbedded with PEG-BPEI creates a biomaterial with extended 72-hour activity to kill bacterial pathogens, disperse biofilms, and reduce inflammatory responses. The biocompatible porous scaffold encourages quick and effective healing because sponge flexibility makes it simple to mold and fit to wounds. The collagen sponge can be covered with other dressing materials allowing the patients to be mobile whenever possible. These attributes will reduce patient pain and discomfort, improving their quality of life. Type I bovine tendon collagen (Sigma C9879) is stirred for 24 hours in 1 M acetic acid. The resulting slurry is transferred to glass forms (30 mm petri dishes) and frozen at -80 °C. Subsequent lyophilization removes excess water and acetic acid. Dehydrothermal crosslinking (heating at 60 °C for 24 hours under vacuum) strengthens the sponge.



Time release properties are enabled by the chemical nature of PEG-BPEI and collagen. Treating collagen with acetic acid disrupts collagen peptides, exposing charged amino acid residues, such as glutamic acid (Glu) whose anionic charge arises from a carboxylate sidechain. The Glu fraction is 11% and is the fourth most abundant amino acid in collagen after uncharged glycine (27%), proline (16%), and hydroxyproline (14%). Hydrophilic PEG-BPEI is easily absorbed by collagen sponges and remains in the sponge via weak electrostatic (Coulombic) interactions between the cationic amines of PEG-BPEI and the exposed anionic carboxylates. However, these electrostatic bonds PEG-BPEI can be converted to covalent bonds using vacuum heating to drive the formation of amide bonds between the amines of PEG-BPEI and collagen carboxylate groups. The extent of conversion is time and temperature dependent. Thus, the fraction of electrostatically bound vs. covalently bound PEG-BPEI can be controlled. This will also affect the release of PEG-BPEI from the collagen sponge. The electrostatically bound fraction will be released quickly into solution while the covalently bound fraction will be slowly released, remaining attached to the sponge until proteases attack the collagen peptides and liberate PEG-BPEI, which itself is not susceptible to protease activity.

BACTERIAL KILLING

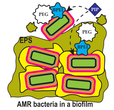
We observe that collagen sponges loaded with 600 Da BPEI are able to kill cultures of *Pseudomonas aeruginosa* PA01. These cells secrete blue green pigments and thus the absence of color indicates a lack of bacterial cells. Strong bacterial growth is observed in media only or collagen only.



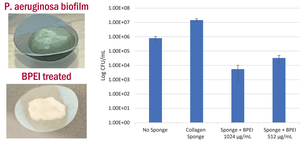
Treatment with 600 DaBPEI and collagen sponges loaded with 600 Da BPEI kill the bacterial cells. These data show that collagen can be dused to deliver the 600 Da BPEI while not impeding activity.

BIOFILM ERRADICATION

Extracellular polymeric substance (EPS) is composed of extracellular DNA, cationic polysaccharide Pel, anionic polysaccharide Psl, and anionic polysaccharide alginic acid, as a barrier against antimicrobial agents. Anionic EPS binds Ag+ ions, cationic polyhexamethylene biguanide (PHMB), and cationic antibiotics. However, when EPS binds PEG-BPEI, the biofilm disperses. This enables biofilm erradication by allowing antimicrobiable agents to reach, and kill, the bacteria.



Biofilms were grown and treated with 600 Da BPEI loaded collagen sponges. The images and data below show that the sponge effectively delivers BPEI and kills the biofilm.



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