Surface Science 648 (2016) 42–46

Contents lists available at ScienceDirect

Surface Science

journal homepage: www.elsevier.com/locate/susc

An affinity adsorption media that mimics heparan sulfate proteoglycans for the treatment of drug-resistant bacteremia

Keith R. McCrea *, Robert S. Ward

ExThera Medical Corporation, 813 Heinz Ave., Berkeley, CA 94710, USA

ARTICLE INFO

Available online 5 November 2015

Keywords:
Drug resistance
CRE
MRSA
ESBL
Affinity adsorption
Biomimetic Surfaces

ABSTRACT

Removal of several drug-resistant bacteria from blood by affinity adsorption onto a heparin-functional media is reported. Heparin is a chemical analogue of heparan sulfate (HS) proteoglycans, found on transmembrane proteins of endothelial cells. Many blood-borne human pathogens, including bacteria, viruses, parasites, and fungi have been reported to target HS as an initial step in their pathogenesis. Here, we demonstrate the binding and removal of Methicillin-resistant *Staphylococcus aureus* (MRSA), Extended-Spectrum Betalactamase *Klebsiella pneumoniae* (ESBL), and two Carbapenem-resistant Enterobacteriaceae (both CRE *Escherichia coli* and CRE *K. pneumoniae*) using 300 μm polyethylene beads surface modified with end-point-attached heparin. Depending on the specific bacteria, the amount removed ranged between 39% (ESBL) and 99.9% (CRE). The total amount of bacteria adsorbed ranged between 2.8×10^5 and 8.6×10^5 colony forming units (CFU) per gram of adsorption media. Based on a polymicrobial challenge which showed no competitive binding, MRSA and CRE apparently utilize different binding sequences on the immobilized heparin ligand. Since the total circulating bacterial load during bacteremia seldom exceeds 5×10^5 CFUs, it appears possible to significantly reduce bacterial concentration in infected patients by multi-pass recirculation of their blood through a small extracorporeal affinity filter containing the heparin-functional adsorption media. This 'dialysis-like therapy' is expected to improve patient outcomes and reduce the cost of care, particularly when there are no anti-infective drugs available to treat the infection.

1. Introduction

The surface chemistry and nanostructure of medical devices are important determinants of biological interactions. Methods of controlling the surface properties of polymers, independent of bulk composition, have application in the development and manufacturing of many disposable devices and prosthetic implants. We have previously reported methods of modifying surface properties of biomedical polymers that rely on surface activity and self-assembly, e.g., for the purpose of enhancing biostability or passive thromboresistance [1–4]. Here we present results of work to develop a therapeutic 'sorbent hemoperfusion device' for broad-spectrum removal of pathogens, toxins and inflammatory molecules from whole blood. We created a biomimetic adsorption media to bind pathogens in large amounts within a single-use extracorporeal filter. Broad-spectrum sorbent hemoperfusion has the potential to treat bloodstream infections early, even before the pathogen is identified. By reducing the intensity and duration of bacteremia or viremia it is expected that patient outcomes will improve and the cost of care will be reduced. Binding to our adsorption media appears to be independent of drug-resistance.

The emergence of drug-resistant pathogens is a growing threat to the healthcare system. Not only are current antibiotics becoming less effective, large pharmaceutical companies are shifting focus from antimicrobial development to more lucrative drug discovery programs such as cancer therapeutics. Although it is recognized that "superbugs" are a major concern, the current market for new anti-infective drugs is relatively small in comparison to their significant regulatory and development costs.

In September, 2013 the U.S. Center for Disease Control (CDC) issued a report on antibiotic-resistant threats [5] in which, the most serious threats are summarized and ranked by hazard levels. A list of 18 drug-resistant bacterial species or strains are classified as 'Urgent', 'Serious', or 'Concerning'. The CDC's definition of the 'Urgent' hazard level is:

> These are high-consequence antibiotic-resistant threats because of significant risks identified across several criteria. These threats may not be currently widespread but have the potential to become so and require urgent public health attention to identify infections and to limit transmissions.

Carbapenem-resistant Enterobacteriaceae (CREs) are one of the three 'Urgent' level threats highlighted in the CDC report. It is estimated that there are currently 9300 cases of CREs each year that leads to 610 deaths annually. The highest profile example of a CRE outbreak occurred at the Clinical Center of the US National Institutes of Health (NIH) in 2011. [6] During this outbreak, 18 patients were infected with CRE

* Corresponding author.
E-mail address: KeithMcCrea@ExTheraMedical.com (K.R. McCrea).

http://dx.doi.org/10.1016/j.susc.2015.10.006
0039-6028/

Klebsiella pneumoniae leading to 6 deaths. The largest outbreak to date occurred at Advocate Lutheran General Hospital in suburban Chicago where 44 patients were infected in 2013. If CREs invade the blood stream, the mortality rate can be as high as 50%. [5] Resistance of CREs to even the strongest available antibiotics leaves clinicians with few treatment options as Carbapenem antibiotics were considered to be the last resort to treat serious gram negative infections. [5] The incidence of hospital-acquired CRE infections has increased significantly over the last 10 years, with a 500% increase in the Southeastern United States since 2008. [7] Currently CRE bacteremias are mostly nosocomial infections, but there is concern that the incidence of community acquired CRE could increase. Today the only strategy available is to reduce the frequency of CRE infections through education and prevention.

Extended-spectrum Betalactamase (ESBL) Enterobacteriaceae and Methicillin-resistant *Staphylococcus aureus* (MRSA) are listed as severe threats in the CDC report. The US incidence of ESBL infections is estimated at 26,000 patients annually, leading to 1700 deaths. [5] Today MRSA is much more common than CRE and ESBL, with an estimated annual incidence of 80,000/year leading to 11,000 deaths. [5] The conventional strategy for combating bacterial infections is to administer active drugs that specifically kill bacteria in the body while minimizing damage to host tissue. This is a major challenge as some of the still effective antibiotics available are quite toxic. For example, vancomycin is nephrotoxic, and the duration of its use has been correlated with acute kidney injury (AKI). [8] Even if new, less toxic antibiotics are successfully developed to address current drug resistance, new superbugs will likely continue to emerge. [9] Clearly, new strategies for combating infection are needed, in addition to drug discovery.

An emerging therapy to combat bacteremia is to remove circulating bacteria by a 'sorption hemoperfusion device'. One approach relies on natural mechanisms that bacterial adhesins utilize in their initial attachment to endothelial tissue during colonization of their human hosts, and subsequent pathogenesis. By engineering artificial polymer surfaces with specific biomimetic binding sites, a high-surface-area extracorporeal affinity adsorption technology can be developed to quickly remove pathogens from whole blood.

In this study, we report on the ability of covalently bound, end-point-attached heparin on ultrahigh molecular weight polyethylene (UHMWPE) beads to remove both drug-susceptible and drug-resistant bacteria. These include CRE *Escherichia coli*, CRE *K. pneumoniae*, ESBL *K. pneumonia* and MRSA. In addition, the simultaneous binding and removal of CRE and MRSA are demonstrated. Depending on the starting concentration, up to 99.9% of the challenge bacteria binds to the adsorption media. A single gram of the particulate adsorption media is shown to have a binding capacity of over 2 million colony forming units (CFU) for *E. coli*. Simultaneous polymicrobial binding data of MRSA and CRE *K. pneumoniae* indicates non-competitive binding, which suggests a different heparin binding sequence for each bacteria. The data demonstrates that an affinity adsorption hemoperfusion device could be used to remove a high concentration of circulating drug-resistant bacteria in blood and thereby become an effective adjunctive or alternative to antibiotic therapy.

2. Materials and methods

2.1. Covalent end-point attachment of heparin and test articles

Ultrahigh molecular weight polyethylene (UHMWPE) beads, with an average diameter of 300 μM were supplied by DSM Biomedical (Berkeley, USA). Pharmaceutical grade heparin and polyethyleneimine (PEI) are purchased from Scientific Protein Laboratories (Waunakee, Wisconsin, USA) and BASF (Ludwigshafen, Germany) respectively. All chemicals used are of analytical grade or better.

Immobilization of heparin onto the beads was performed as described by Larm et al. [10] Briefly, the UHMWPE surface was heparinized using the general procedure described below. The surface was etched with potassium permanganate in sulfuric acid to hydrophilize the beads. Reactive amino functions are introduced by treatment with polyethyleneimine (PEI). End-point attachment (EPA) to the aminated surface of native heparin is done by reductive amination, by reacting the aldehyde group in the reducing terminal of partially, nitrous degradation heparin. The resulting PE-beads, with covalently end-point attached heparin, were then sterilized with ethylenoxide (ETO) and outgassed.

The sterilize beads (0.6 g) were packaged in 2.5 ml test filter syringes (Mobicol, Germany) with 90-micron top and bottom retaining plates. A total of three filter syringes were prepared for each pathogen.

2.2. Surface heparin loading and activity

The surface loading of heparin was determined using the 'MBTH method' [10].

To verify that the covalent end-point attached heparin maintained its ATIII activity, the surface was characterized using Kinetichrome™ Heparin Anti-Xa Activity Kits (Provision Kinetics, Arlington WI). The general procedure for the Anti-Xa test is as follows. A measured quantity of heparin beads is first suspended in PBS buffer at 37 °C. ATIII in buffer is then pipetted into the suspension and allowed to form a surface bound ATIII/heparin complex. An excess of Anti-Xa is then added to the suspension and only a portion binds to the surface ATIII/Heparin complex. Finally, a chromogenic Anti-Xa substrate (which is a peptide with affinity to anti-Xa) is then added to the solution to react with the residual Anti-Xa in solution. The concentration of the Anti-Xa/Substrate is then determined using a UV/Vis spectrometer and the heparin surface activity is calculated.

2.3. Microbiology

The microbiology testing was performed at Antimicrobial Test Laboratories (ATL). For this study, the removal of high concentrations of bacteria suspended in defibrinated horse blood (Hemostat Laboratories) was tested. The MRSA strain was ATCC 33592. The Enterobacteriaceae tested included drug susceptible *E. coli* ATCC 8739 and Carbapenem-resistant *E. coli* ATCC BAA-2469, drug susceptible *K. pneumoniae* ATCC 13883, Carbapenem-resistant *K. pneumoniae* ATCC BAA-2146, and ESBL *K. pneumoniae* ATCC-770603. The bacteria were cultured using standard methods and diluted in defibrinated horse blood. The targeted CFU/mL concentration was typical for antimicrobial testing and ranged between 10^5 and 10^6 CFU/mL. The filter syringes were primed/deaired with saline. An aliquot of blood with a bacterial concentration of ~10^6 CFU/mL was then passed through the filter syringe by gravity flow (N = 3), collected, and analyzed. Additional aliquots (N = 3) were saved to enumerate the starting concentration. The starting aliquots and filtrates were neutralized and enumerated simultaneously, and reduction in bacterial numbers determined (CFU/mL).

For the polymicrobial binding study, MRSA and CRE *K. pneumoniae* were independently cultured as outlined above. Both bacteria were then diluted into a single sample of defibrinated horse blood. Aliquots were then either passed through the filter syringes (N = 3) or retained for starting concentration (N = 3) analysis as described above.

3. Results

3.1. Loading and activity

MBTH analysis indicated a heparin loading of 2.0 mg per gram of beads. Activity analysis by Anti-XA assay determined a heparin activity of 0.3 U/mg of beads. No leaching of heparin from the surface was detected, confirming that the heparin is covalently attached.