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Dialysis Membrane Manipulation for Endotoxin Removal

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1. Introduction

In the dialysis clinic, water is an essential vehicle to deliver life-saving treatment to patients suffering from varying degrees of kidney failure, both acute and chronic. Clean water is vital, as the key ingredient used to prepare hemodialysis fluid (dialysate solution), and on-line generation of substitution fluid for hemodiafiltration. Generally all fluids used to treat patients suffering from kidney failure may come into contact with the blood of the patient, whether directly or indirectly (across a membrane), and theoretically could transport contaminants resulting in a negative impact on patient health. Of the microbiological contaminants found in water, endotoxin is given considerable attention, given its difficulty for removal and inactivation from water and water distribution systems (Smeets et al., 2003; Perez-Garcia & Rodriguez-Benitez, 2000) and its inherent pyrogenicity (G. Lonnemann, 2000).

Endotoxins are found in all gram-negative bacteria, although slight differences in chemical structure are found between varying bacterial strains. The term endotoxin is typically used to describe a complex of protein and lipopolysaccharide (LPS) molecules found in the outer cell wall of gram-negative bacteria, that either slough off during growth, or are released upon cell lysis. Endotoxin and lipopolysaccharide are typically used interchangeably in literature, although in clinical discussion the term endotoxin is most often used, as it is the metric used to monitor water and dialysis fluid quality. Lipopolysaccharide is a vital component of the outer membrane of gram-negative bacteria, providing numerous physiological functions and comprising nearly 75% of the bacterium outer surface area (Raetz, 1991). Lipopolysaccharides consist of three components: a long heteropolysaccharide chain (O-specific chain) which represents a surface antigen; a core oligosaccharide; and a lipid component termed lipid A used as an anchor in the outer cell membrane (Rietschel et al., 1994; Gorbet & Sefton, 2005). Molecular weights of most lipopolysaccharides are 10 – 20 kDa; however, due to their amphiphilic nature, LPS molecules can form aggregates (100 – 1000 kDa) which are too large to pass through dialysis membranes. It has been shown that components of lipopolysaccharide (lipid A) are able to pass through dialysis membranes, can elicit a pyrogenic response (Naveh-Many et al., 1999), and contribute to long-term morbidity and inflammation (H. Schiffl, 2000; Raj et al., 2009).

Lipid A is the most conserved component of lipopolysaccharide throughout all gram-negative bacteria, and as such is responsible for the majority of the pyrogenic activity. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimmer connected to saturated

fatty acid chains; variability within the composition of the fatty acids will determine the toxic property of lipid A, as well as play a role in resistance to host antimicrobial factors and avoiding recognition from specific components of the host immune system (Bland et al., 1994; Gunn, 2001; Qureshi et al., 1999). The O-specific side chain component of LPS is responsible for complement activation and contributes to fever and hypotension, as well as binding to endotoxin recognition molecules within the body (Valvano, 1992; Bailat et al., 1997). Once within the body, LPS tend to be found at higher concentrations within the spleen and liver (uptake by phagocytosis) where they are cleared from the body (Haeffner-Cavaillon et al., 1998).

Dialysis patients are typically exposed to 90 – 120 liters of dialysis fluid per treatment, which equates to an annual exposure of 20 – 30,000 liters (Weber et al., 2004; I. Ledebo, 2002). With constant exposure to large amounts of fluid, the opportunity for a dialysis patient to experience an inflammatory or pyrogenic reaction due to contamination within the dialysis fluid is increased. For hemodialysis, fluids that are used for treatments do not have to be sterile; however, the lower the microbial concentration, the lower the risk of patient reaction. Because of this risk, regional regulatory boards have implemented limits to the total microbial count that can be present in fluids that are to be used in dialysis treatments. However, even if water treatment systems are in place, contamination is still a possibility and a risk. Dialysis fluid used for clinical treatments may become contaminated from either the source water, the dialysate concentrate, or from the water distribution system. Due to the ubiquitous nature of biofilm, and its propensity to generate endotoxin, this problem affects not only hemodialysis, but all extracorporeal therapies (Kanagasundaram et al., 2009).

Regardless of the treatment processes used to create water for dialysis fluid, the final opportunity to remove microbial contaminants from the fluid path prior to patient exposure is the dialysis membrane contained within the dialyzer. Dialysis fluid comes into direct contact with this membrane, and due to transmembrane pressure differences and the permeability of the filter, especially for high-flux dialyzers, the potential for dialysis fluid to enter the blood compartment and return to the patient is significant (N. Hoenich, 2007). It is this final barrier, the dialyzer membrane, where the last opportunity resides to remove endotoxins from solution (Weber et al., 2009), by means of membrane manipulation. The aim of the following research is to achieve a more thorough understanding of how endotoxin interacts with various physical characteristics of the dialysis membrane, and how to exploit these interactions to increase endotoxin removal from dialysis fluid.

2. Membrane manipulation for endotoxin removal

The degree of contaminated dialysis fluid including bacteria, bacterial fragments and endotoxin that may enter the bloodstream of a patient during treatment depends largely upon the porosity and other physical characteristics of the membrane being utilized. Back-filtration is based upon geometrical and permeability properties of the hollow fiber membrane, and cannot be avoided in high-flux hemodialysis (Ofsthun & Leypoldt, 1995). However, both physical and chemical means can be used to prohibit endotoxin from crossing the membrane, by removing it from solution and holding it within the dialyzer membrane. Numerous studies have been performed to determine the properties of hemodialysis membranes that best manipulate the transfer of endotoxin, by removing it from the dialysis circuit (Canaud et al., 2000; Lonnemann et al., 2001). Surface treatments,

polymer modifications, as well as chemical changes to the membrane composition, have been investigated to ascertain their influence on preventing or minimizing endotoxin and bacterial fragment flux across the membrane. Some studies have shown that even the choice of sterilization modality may have an impact on the membrane, and affect the ability to retain endotoxin (Gomila et al, 2006; Krieter et al., 2008). It is necessary to understand how a particular dialysis membrane interacts with endotoxin and other dialysis fluid contaminants, as the membrane is the last barrier to the patients' blood. Endotoxins (and other types of microbial contamination) are removed from dialysis fluid mainly by one of two methods: filtration and adsorption. Studies have shown that both methods of endotoxin removal occur during dialysis treatment (Osumi et al., 1995). Understanding endotoxin interactions with various membrane surfaces is imperative in order to orchestrate changes that will have a positive impact on endotoxin removal. The end goal of all endotoxin research is to limit patient exposure, in hopes of reducing the chance for pyrogenic reactions, inflammation, and shock.

2.1 Membrane geometry

Prior to synthetic membranes occupying the majority of the dialysis filter market, cellulosic membranes were the predominant choice for manufacturers. Cellulose was a material that could be modified to improve its biocompatibility; however its geometrical manipulation was limited due to the production process. As membrane materials progressed from cellulose-based to synthetic, numerous adjustments could be made to the physical characteristics of the membrane by relatively simple manufacturing process changes.

One of the most direct methods to inhibit endotoxin is to change the material structure of the membrane itself, as the physical attributes of the membrane will perhaps have the greatest effect on endotoxin removal. Typically a thin-wall membrane is not as robust as a thick-wall membrane, in terms of endotoxin adsorption, since a thicker membrane can offer more surface area for the endotoxin to come into contact with. A thicker membrane provides a longer path for the endotoxin to maneuver through, before it reaches the blood circuit. An important characteristic of this path from outer membrane surface to inner membrane lumen is tortuosity, the curving path that the endotoxin must follow in order to reach the blood compartment of the fiber membrane (Osumi et al., 1995). As tortuosity is increased, the greater the chance the membrane has at prohibiting the passage of endotoxin. Membrane geometry changes can lead to differences in the adsorptive capacity for endotoxin (Vanholder & Pedrini, 2008; Vaslaki et al., 2000) and for bacteria (Waterhouse & Hall, 1995).

Changes in membrane permeability are controlled to enhance convective transfer, which targets middle molecular removal of species such as β_2Microglobulin and vitamin B_{12}. As dialysis membranes are pushed for more convective removal ability, the opportunity for endotoxin trans-membrane flux increases due to the higher chance of back-filtration. Future membranes designed to address middle molecule removal by increasing internal filtration (Mineshima et al., 2009) will not improve on patient inflammation (Kerr et al., 2007) unless membrane geometry is modified to improve endotoxin removal.

The effect of membrane thickness and permeability on endotoxin removal was studied by testing various membrane configurations, and by observing their ability to restrict contaminant flux. Synthetic membranes for testing were manufactured with specific geometries to observe their performance relative to a control. Fiber geometries tested included low flux, high flux, thin wall, thick wall, macrovoid, and a control. Characteristics

of each test membrane produced for this study are listed below in Table 1. The endotoxin challenge solution used was comprised of a 1:1 mixture of bacterial culture filtrates of Stenotrophomonas maltophilia (ATCC 13637) and Pseudomonas aeruginosa (ATCC 27853), both common water organisms. Cultures of each microorganism were ultra-sonicated to lyse the cells and release the endotoxin fragments, then filtered using a sequentially-decreasing process resulting in a final 0.2 µm filtration step.

Fiber Type	Fiber ID, µm	Fiber Wall, µm	Fiber Kuf, mL/hr*mmHg	Material, Fiber type
Control	185	35	200	Polysulfone (PS), asymmetric
Low Flux	181	39	48	PS, asymmetric
High Flux	182	37	522	PS, asymmetric
Thin Wall	187	24	231	PS, asymmetric
Thick Wall	184	44	190	PS, asymmetric
Macrovoid	212	33	130	PS, dual skin

Table 1. Fiber membrane geometries

Bacterial culture filtrates were used instead of purified LPS to produce challenge material that would closely resemble clinical experiences. Membranes were tested for their ability to restrict the passage of endotoxin across the membrane by either filtration or adsorption, by using a test setup (Figure 1) that focused on the diffusive and convective aspects of hemodialysis. The first setup focuses on diffusive testing, whereby the counter current flow mimics a typical hemodialysis treatment. The second setup in the test forces fluid to flow through the membrane, testing the filtration capacities of each fiber membrane. Initially, the test setup involved connecting the test dialyzer to a recirculating loop, where it could be connected to a peristaltic pump to control the flow of fluid on the blood loop. The dialysis fluid loop consisted of tubing that was connected to a reservoir, which allowed fluid to be recirculated through the dialysis fluid compartment, via another peristaltic pump. This reservoir held 1 liter of challenge solution, containing bacterial culture filtrate mixed into saline to produce an endotoxin concentration of 400 +/- 50 EU/mL. The blood loop

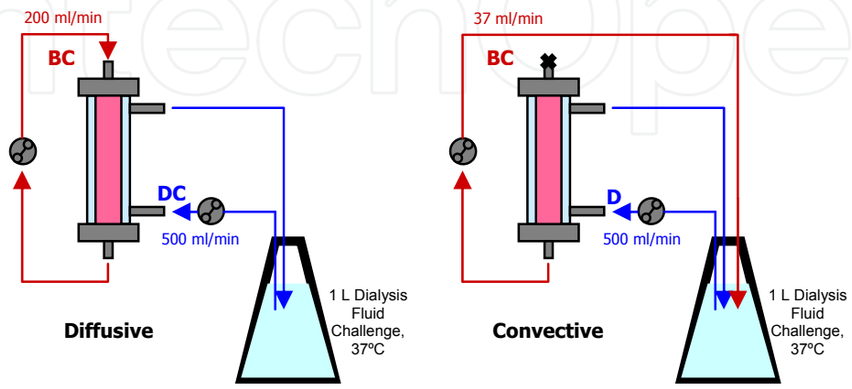

Fig. 1. Endotoxin challenge test setup, showing the blood loop and dialysis fluid loop flowrates for both diffusive and convective testing.

peristaltic pump was set at 200 mL/min to recirculate the enclosed saline, while the dialysis fluid loop pump was set at 500 mL/min to recirculate the endotoxin challenge solution from the reservoir, through the dialyzer, and back. This test setup was maintained inside an incubator set at 37 C. Prior to starting the test, the dialyzer (both blood loop and dialysis fluid loop) were primed with sterile saline, to rinse out any residual endotoxin that may be contained within the test setup. Once the pumps were initiated, a timer was started and samples were taken from both the blood loop and dialysis fluid loop at the following times: 0, 7, 15, and 60 minutes. Following the 60 minute sample, the test setup was changed according to Figure 1, so that dialysis fluid was forced across the membrane into the blood loop, and back into the challenge reservoir. Again, samples were taken following the start of the second half of the experiment at 67, 75, and 120 minutes. Samples were kept refrigerated at 4 C until ready to be analyzed for endotoxin content.

Endotoxin activity was measured using a kinetic turbidimetric Limulus Amoebocyte Lysate (LAL) assay from Charles River Labs. Each sample was measured in duplicate, along with a positive control to verify recovery within the sample. Results from these tests are shown in Figure 2, with curves to represent both blood side and dialysis fluid side endotoxin concentrations. The data were able to show that only the control and thick wall membranes were able to contain the endotoxin in the blood side below < 0.1 EU/mL; all other membrane types allowed measurable amounts of endotoxin to cross into the enclosed blood loop circuit. Also, the results indicate that some membranes perform better at prohibiting

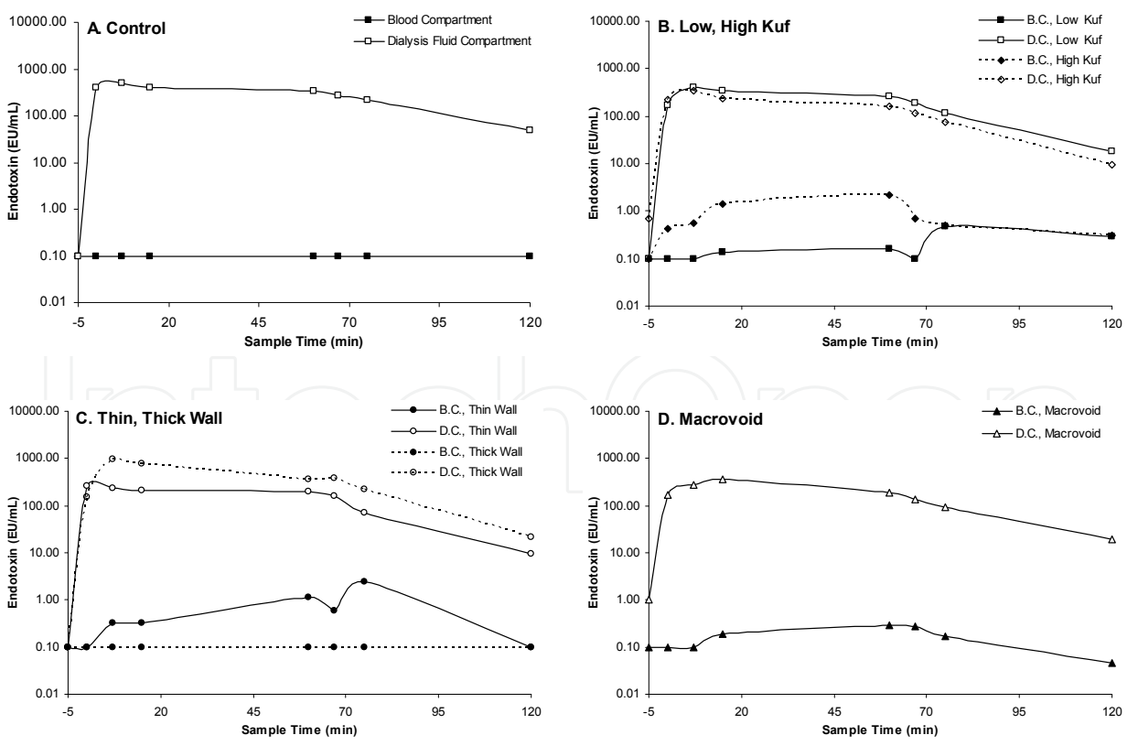

Fig. 2. Endotoxin challenge results for test simulations (all tests, n=3). a) Control membrane; b) Low and High Kuf membranes; c) Thin, Thick Wall membranes; d) Macrovoid membrane.

diffusive endotoxin flux than convective endotoxin flux, and vice versa. In particular, the high flux and thin wall membranes allowed the highest amounts of endotoxin into the blood compartment, as these membranes present the shortest tortuous path (thin wall) and the highest potential for back-diffusion along the membrane (high flux). These findings suggest that asymmetric, thick membranes are better suited at removing endotoxin from solution, as well as allowing endotoxin and other pyrogenic substances from crossing the membrane and contaminating a patient blood-flow.

Not only is a thick fiber good at preventing endotoxin contamination to the patient, but the outer or inner membrane surface of the fiber membrane are imperative at preventing trans-membrane endotoxin flux. Pore size distribution, or surface morphology, is a strictly controlled process in how to determine the flux of the dialyzer, and in allowing molecules of a certain size to either pass through the membrane surface, or be retained. Dialysis membrane pore sizes are not manufactured with endotoxin transfer in mind; however the pores play a critical role in regulating trans-membrane passage. Membrane thickness, structure, and surface morphology influence the membranes' ability in preventing endotoxin transfer through geometrical means, mostly by way of filtration. However, chemical changes within the membrane can also add benefits towards increased endotoxin performance, with respect to removal by way of adsorption.

2.2 Membrane surface changes

Similar to how membrane geometry directly affects the endotoxin sieving ability, the surface properties of membranes will govern the adsorption capacity for endotoxin by providing a suitable surface for endotoxin and endotoxin fragments to adsorb by utilizing their amphiphilic nature. The hydrophobic lipid A moiety is typically attracted to hydrophobic surfaces, however the hydrophilic polysaccharide component will also allow for adsorption to hydrophilic surfaces (Takemoto et al., 2003). Recent studies have shown this to be the case, particularly for ultrafilters which remove endotoxin by way of adsorption through ionic and hydrophobic interactions (Vaslaki et al, 2000). When adjusting the polymer ratios of dialysis fiber membrane, chemical changes as well as physical changes can be produced. The overall geometry of the fiber can be manipulated by the polymer ratio – whether the fiber is based upon a sponge structure, whether the membrane possesses macrovoids, or whether the membrane exhibits one skin or two.

Synthetic dialysis membranes from polysulfone are typically hydrophobic in nature, thus adding to their ability in being a good hemocompatible membrane for blood interaction. When manufacturing such membranes it is necessary to utilize a hydrophilic polymer that allows for the membrane to "wet"; to possess hydrophilic regions or properties. By simply adjusting the ratio of hydrophobic polymer (polysulfone) to hydrophilic polymer (polyvinylpyrrolidone or PVP), the membrane hydrophobicity composition can be changed significantly. A membrane that incorporates varying regions of hydrophobicity may be able to exploit the amphiphilic nature of endotoxin, resulting in numerous opportunities to remove it from dialysis fluid via adsorption (Maitz et al., 2009). Enhanced endotoxin removal has been shown feasible by increasing the surface polarity of the membrane (Rimmele et al, 2008). Polysulfone membranes typically exhibit a net negative surface charge, termed zeta potential, which may aid in their ability to remove endotoxin through adsorption (Mares et al, 2009; Shao et al., 2007). Similar behaviors are witnessed when endotoxin solutions are kept in glass containers; the glassware adsorbs a portion of the endotoxin, thus necessitating the glass to be cleaned or "depyrogenated."

A special consideration to not overlook is where dialysis clinics practice reuse; numerous studies have investigated the effect of reprocessing agents on dialysis membrane performance and the resulting effect on endotoxin retention (Teehan et al., 2004; Sundaram et al., 1996). For those using bleach as a disinfectant and sterilizing agent, its use has to be taken into account as repeated exposure to membranes have been shown to increase solute clearance, as well as increase the net negative charge, thus increasing hydrophilicity (Shao et al., 2007).

Polymer mixes were varied for test membranes, to study the endotoxin removal performance based upon chemical surface changes. Membranes, manufactured for this study, included those composed of high and low PVP content, a membrane consisting of a polymer mixture (polysulfone, PEG) and a membrane exposed to bleach – to determine how fiber surface differences affect the overall ability to remove endotoxin from solution. Also, membranes were tested using a fluorescent-labeled endotoxin to show the distribution of endotoxin within the membrane, modified from prior studies (Hayama et al., 2002). The experimental test setup was similar to that used to test fiber geometries – the test environment temperature, blood loop and dialysis fluid loop flow rates, endotoxin challenge concentration, and sample analysis method were all kept constant as described previously.

An additional test was performed for these membranes, using a labeled endotoxin to identify where the endotoxin molecule is removed from solution within the fiber membrane. To accomplish this, miniature dialyzers were constructed of small polycarbonate housings and T's to create a membrane dialyzer that could filter a smaller volume of fluorescent challenge solution, a mixture of Alexa Fluor 568 (Invitrogen, Carlsbad, CA) in saline to produce a concentration of 2,000 EU/mL. Membranes were challenged using a test setup similar to the diffusive setup used for the endotoxin challenge simulations, utilizing a challenge fluid flow on the dialysis fluid side of 5 mL/min, and a countercurrent fluid flow of saline on the blood side of 2 mL/min. Upon completion of the diffusive test, fibers were extracted from the miniature dialyzer housings and fixed into freezing media. A cryostat was used to slice the membrane samples into 10 um sections, which were then imaged using a microscope fit with a resorufin filter to observe the distribution of endotoxin on the membrane.

Membrane permeability tests (Figure 3) were able to show how the bleached membrane, the high PVP content membrane, as well as the polymer mixture membrane, all allowed significant amounts of endotoxin to cross into the blood loop compartment. Fluorescent imaging revealed that for all membranes the majority of the endotoxin (highest intensity) was bound at either the inner or outer lumen of the fiber; also, differences could be seen in the distribution of endotoxin inside the bulk of the membrane, with some membranes showing high intensity, while others hardly could be imaged due to the low intensity of the endotoxin (Figure 4).

These results indicate that chemical changes to the membrane can be manipulated in order to direct the preferential adsorption of endotoxin in a discreet region of the fiber, such as the outer lumen or inner lumen. These types of changes may be beneficial depending upon the intended use of the membrane, such as dialysis or ultrafiltration. Researchers have postulated that the region within the membrane where endotoxin is removed is important, as it has been shown to induce cytokine activation in blood within a dialyzer when bound adjacent to the lumen surface (Okamoto et al., 2004). These test results also provide insight into the nature of where and how endotoxin is bound or adsorbs to the membrane, furthering our understanding in how to remove it from solutions.

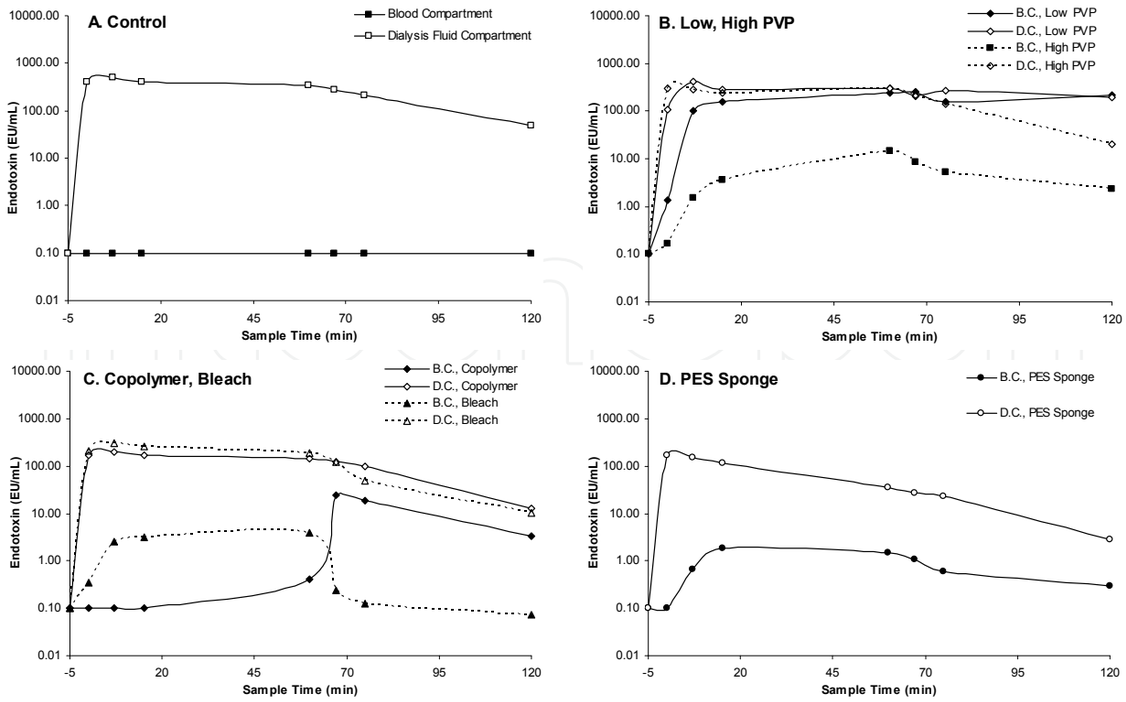

Fig. 3. Endotoxin challenge results for test simulations (all tests, n=3). a) Control membrane; b) Low, High PVP membranes; c) Copolymer, Bleach membranes; d) PES Sponge membrane.

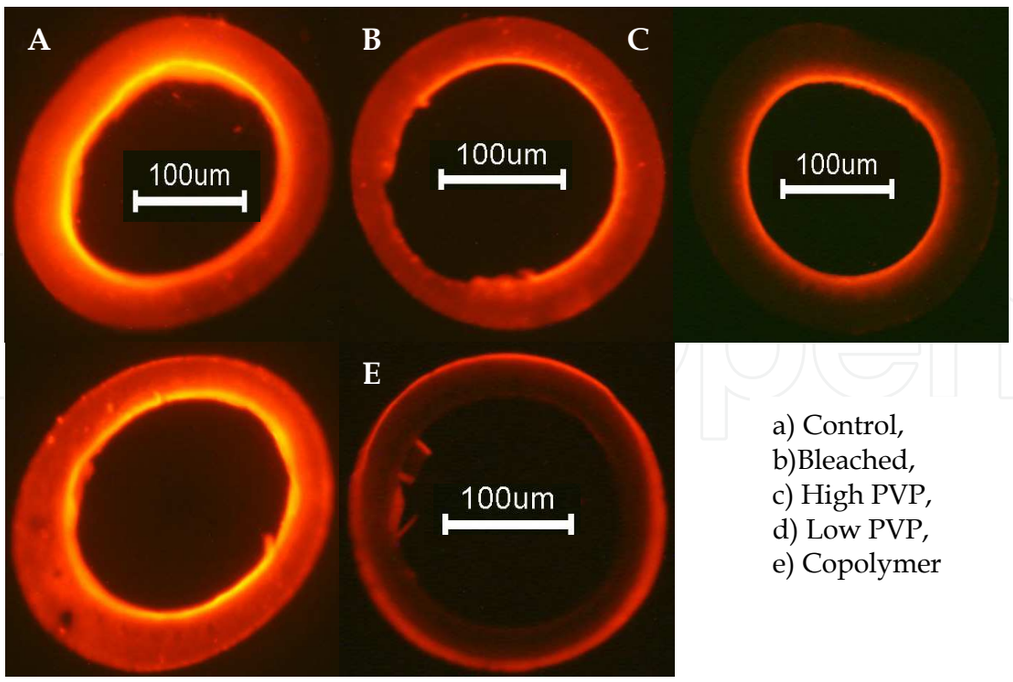

Fig. 4. Fiber membrane sections showing the distribution of fluorescent-labeled endotoxin (Alexa Fluor 568, imaged using a Resorufin filter).

2.3 Membrane material

The type of membrane structure possible, which directly relates to the endotoxin removal mechanism (filtration, adsorption), is largely governed by the material used to produce the membrane. Polysulfone is currently the most common membrane material in the chronic dialysis market, but is only one of several choices available to patients and nephrologists. Dialysis membranes may also be produced from materials such as cellulose, poly methyl methacrylate (PMMA), polyester-polymer alloy (PEPA), polyethersulfone, polyamide, and cuprophane. Use of cuprophane membranes modified with vitamin E (Girndt et al., 2000), highlight the potential for modifications of specific membrane materials. Significant research has been produced to show superior performance for endotoxin removal by synthetic membranes (Yamamoto & Kim, 1996; Nube & Grooteman, 2001); however, research has also exploited differences in the capacity for endotoxin between membranes of the same material, indicating that slight differences in the manufacturing and finishing processes may have a significant impact on the ultimate performance (Opatrny Jr. et al., 2006). On the contrary, studies have shown that in some instances it is difficult to show a clinical benefit when comparing differing membranes (Boudville et al., 2009; Urena et al., 1992).

The effect of various fiber membrane materials on endotoxin retention was studied by testing polysulfone, cellulose triacetate, and polyethersulfone dialysis membranes using endotoxin permeability studies. An endotoxin challenge of 400 ± 50 EU/mL was created by spiking sterile saline with bacterial culture filtrates of a 1:1 mixture of P. aeruginosa (ATCC 27853) and S. maltophilia (ATCC 13637). Simulation experiments were conducted to assess endotoxin transfer under both diffusive and convective conditions, with sterile saline used to model blood in the blood-side circuit. Flow rates used were similar to prior studies (Q_B = 200 mL/min, Q_D = 500 mL/min) and the experimental temperature was kept constant at 37 C. Samples were taken at times similar to prior studies, and were analyzed using a kinetic turbidimetric LAL assay with a detection limit of 0.1 EU/mL.

The results from these studies (Figure 5) show sponge-structure polysulfone performed the best at prohibiting endotoxin to cross the membrane, under diffusive and convective hydraulic conditions. Cellulose triacetate and polyethersulfone both allowed endotoxin to cross into the inner lumen space of the membrane; it is unclear if these results are indications to limits of endotoxin removal regarding the fiber lumen (pore size) or lumen surface adsorption. Material choice will dictate what type of endotoxin removal will predominate – filtration or adsorption – as certain materials will produce specific structure geometries when undergoing the manufacturing process.

Also, material choice will determine if a membrane is a good candidate for a particular coating or surface treatment to be applied; to aid in either creating a more biocompatible surface, or to enhance the endotoxin retentive properties.

2.4 Membrane coatings

Membrane coatings or surface treatments are not usually found on general use dialyzers; rather, they are used to add enhancements to dialyzers to better help a niche group of patients – whether the need is improved biocompatibility, better anticoagulation, or a reduction in circulating inflammatory cytokines. Surface treatments or coatings could be used to remove endotoxin; however, no coatings for dialyzer membranes (to date) have been produced to directly address endotoxin adsorption or removal. A coating which could produce a mixture of hydrophobic and hydrophilic regions would increase the adsorptive capacity of the membrane to retain circulating endotoxin – and at the same time may aid in

filtrative endotoxin removal. Coatings applied to the membrane post-production should be designed to not decrease/impede any solute clearance performance of the membrane, or inhibit membrane performance in any way.

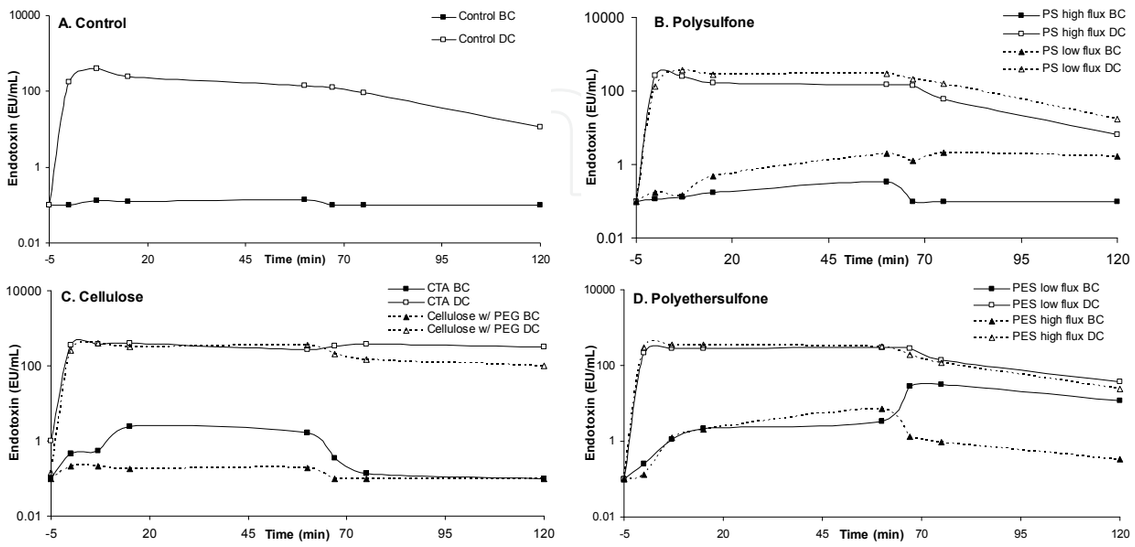

Fig. 5. Endotoxin challenge results for test simulations (all tests, n=3). a) Control membrane (PS); b) Polysulfone, low-flux and high-flux; c) Cellulose, CTA and PEG coated; d) Polyethersulfone, low-flux and high-flux.

Charcoal suspension has been tested as a potential adsorbent for diaysis, although it has been shown to induce platelet activation (Kramer et al., 2000). One significant area of research on endotoxin adsorption is for septic patients. Numerous techniques have been tested to remove circulating inflammatory cytokines from patient blood, as well as remove endotoxin. Polymyxin-B has been studied as an endotoxin binder, due to its affinity to the lipid A component and as it disrupts the permeability of cytoplasmic membranes of Gram negative bacteria (Uriu et al., 2002; Jaber & Pereira, 1997; Tani et al., 1998). Polyethylenimine has been researched as a potential endotoxin adsorbent, and may be more compatible than other types of resins in similar applications (Mitzner et al., 1993). The most prevalent treatment is to use apheresis and hemoperfusion, removing specific targets by resins or coated fibers (Ronco et al., 2000; Szathmary et al., 2004; Yaroustovsky et al., 2009; Umgelter et al., 2008). Some of these endotoxin-specific adsorbents that have been proven effective may come with deleterious side effects (Steczko et al., 1999; Tani et al., 1998).

Efficacy of a potential endotoxin-specific coating application was investigated by treating standard dialysis membranes with two specific polysaccharides (neutral and positive charged chitosan), a tri-block copolymer, and by using a bleach rinse. These modified membranes were tested for their endotoxin retention capacity by using the endotoxin simulation procedure described previously, with the same flow rates (Q_B = 200 mL/min, Q_D = 500 mL/min) and an endotoxin dialysis fluid challenge of 440 ± 55 EU/mL. However, the duration of the experiment was extended to 6 hours (3 hrs. diffusive, 3 hrs. convective) to observe if any plateau of endotoxin filtration or adsorption were to occur. Sampling from the blood and dialysis fluid circuits occurred at the following times (minutes): -5, 0, 7, 15, 30,

and every 30 minutes afterwards until 3 hours had transpired. Samples were analyzed for endotoxin activity using a kinetic turbidimetric LAL assay with a detection limit of 0.1 EU/mL.

Results obtained from the test dialyzers with membrane coatings are shown in Figure 5, showing the endotoxin profile curves for the full 6 hours of testing. Samples analyzed from the chitosan and +chitosan membranes exhibited inflated spike recovery samples, indicative of false positive readings. It is likely that polysaccharide leaching out of the coating and into the recirculating saline solution in the blood compartment compromised the integrity of the samples. It is likely that the actual endotoxin crossing into the blood compartment for the chitosan and +chitosan samples is lower, however the overall endotoxin reduction in the dialysis fluid compartment did not justify repeating the tests using a buffering agent to mask the effects of the polysaccharides. The findings suggest that while all of the investigative treatments did enhance the removal of endotoxin, some were more successful than others (chitosan coatings<control<bleach treated<polymer coating).

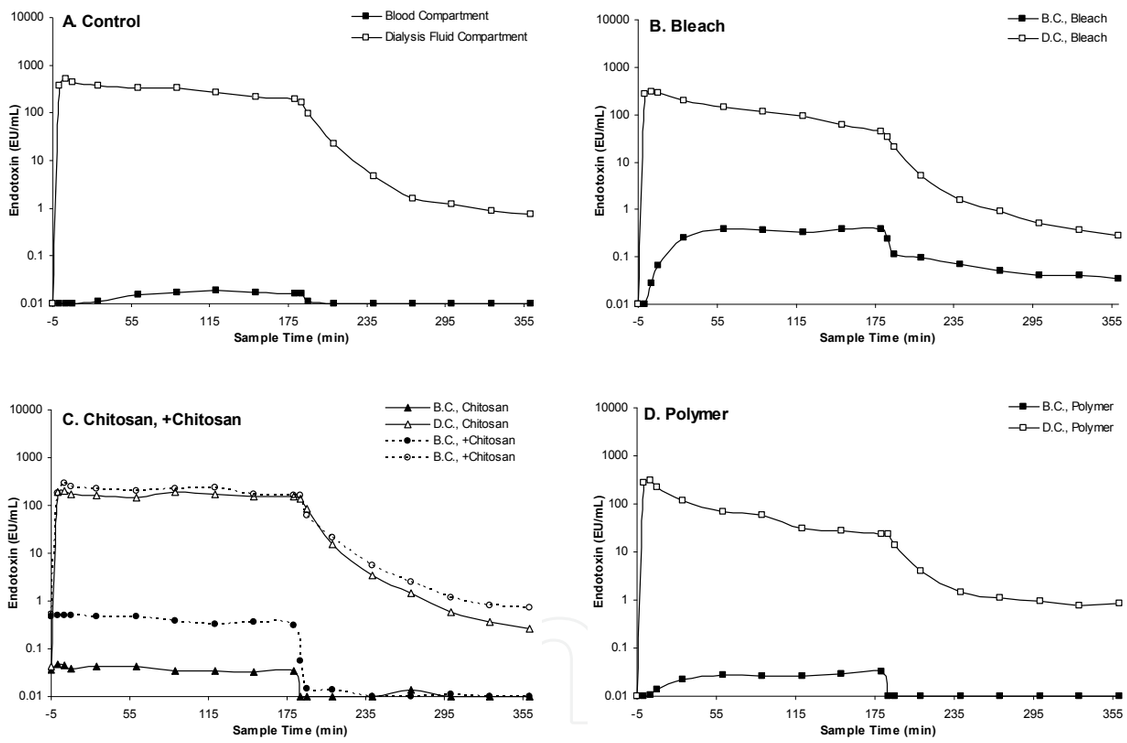

Fig. 5. Endotoxin challenge results for test simulations (all tests, n=3). a) Control membrane; b) Bleach treated membrane; c) Chitosan, +Chitosan coated membranes; d) Polymer coated membrane.

Follow-up experiments were conducted to further observe the ability of each membrane coating at adsorption of endotoxin by exposing each test membrane to a semi-static adsorption environment. Fiber membranes were extracted from test dialyzers, placing 35 cm^2 of each fiber type into a series of 50 mL conical tubes. Each tube was filled with 50 mL of sterile saline, and then spiked with varying amounts of endotoxin resulting in a gradient of concentrations for each membrane type (1, 2.5, 5, 10, 20, and 50 EU/mL). These tubes

were then fixed to a rotor in a 37 C environment, which provided thorough mixing for the test duration of 72 hours. Samples were taken at set intervals, and analyzed immediately using the kinetic turbidimetric LAL assay with a detection limit of 0.01 EU/mL. Data gathered from these tests were used to generate adsorption isotherms for each membrane, shown in Figure 6. These plots indicate that the polymer coated membrane removed more endotoxin, and at a higher rate, than both the bleach treated and control membranes. For all three test membranes, adsorption rates for the 1 and 2.5 EU/mL concentrations reached a plateau at about 60 hours, although at 72 hours the 20 and 50 EU/mL concentrations were still showing measurable adsorption of endotoxin. The adsorption rates were calculated as follows: control – 0.15 ml/cm^2*hr; bleach – 0.11 ml/cm^2*hr; polymer – 0.30 mL/cm^2*hr. The results from the polymer coated membrane are promising, given that the adsorption rate of endotoxin was twice that of the control. These findings suggest that endotoxin-specific coatings for dialysis or ultra filtration membranes are a theoretical possibility, and may aid in other aspects of membrane performance.

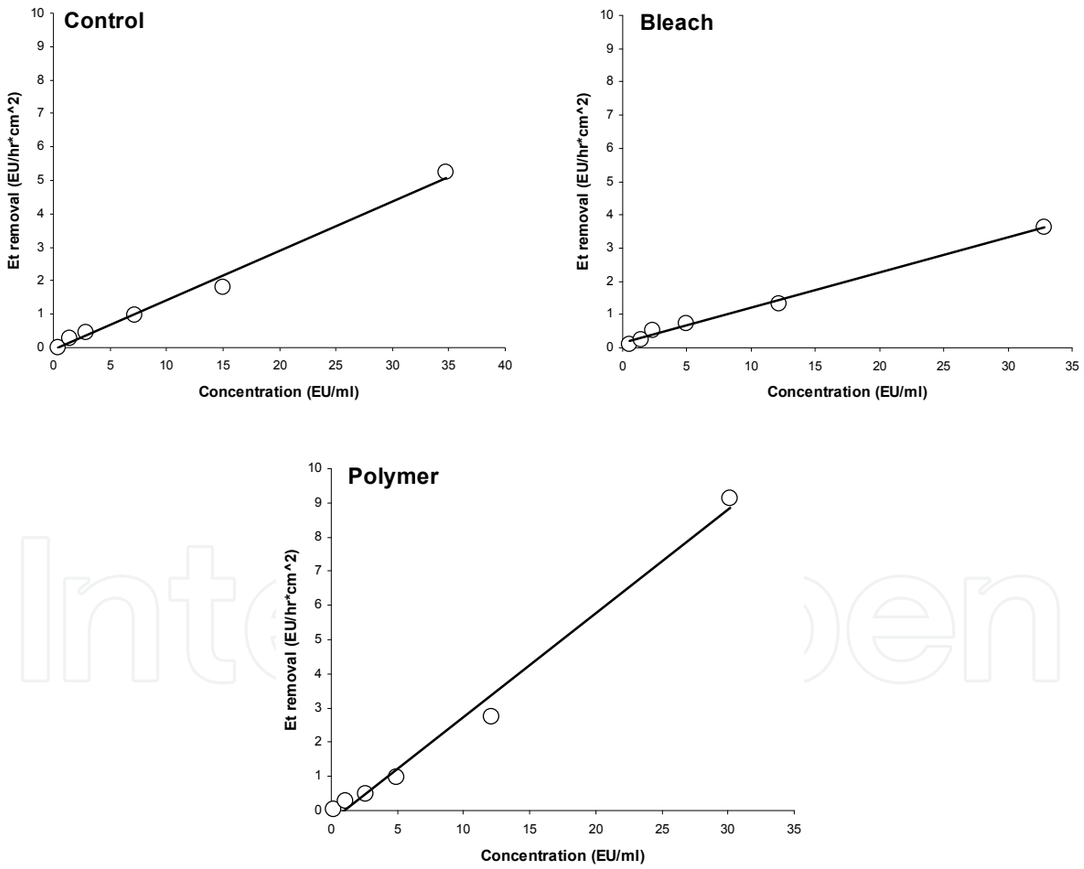

Fig. 6. Adsorption isotherm curves calculated for control, bleach treated, and polymer coated membranes. Curves were calculated from endotoxin challenge solutions of 1, 2.5, 5, 10, 20, and 50 EU/mL. Correlation coefficients for the control, bleach treated, and polymer coated membranes were $r^2 = 0.991$, $r^2 = 0.994$, and $r^2 = 0.989$, respectively.

3. Discussion

Patient health, as it relates to hemodialysis treatment, is of the utmost importance as the mortality and morbidity of ESRD patients is typically high. A strong push recently has been to move to the use of ultrapure dialysate in clinics, as studies have shown correlations between improvements in markers of inflammation and the use of dialysis fluid meeting the criteria of <0.1 CFU/mL and <0.03 EU/mL (Lonnemann & Kock, 2002; Ledebo, 2007a; Schindler, 2009; Schiffl et al., 2002). As some studies have shown that typical endotoxin concentrations are higher in dialysis fluid than RO water entering the hemodialysis machine (von Sengbusch et al., 1993), the ultrapure criteria of the dialysis fluid needs to be created just prior to the patient receiving treatment. One method to produce and ensure the production of ultrapure dialysis fluid is to place an ultrafilter in the hydraulic path between the patient and the dialysis machine, as this filter will have the greatest impact to the final dialysis fluid solution prior to entrance into the dialyzer (Oliver et al., 1992; Schindler et al., 2004).

Studies in the literature have revealed significant removal rates for ultrafilters in vitro, typically >99% for endotoxin (Oliver et al., 1992; Krautzig et al., 1996), with log reduction values >3 desirable (Tsuchida et al., 2009). Ultrafilters are commonly used in HDF, where two filters are typically used in series to guarantee sterile fluid production – manufacturers of ultrafilters state that sterility of substitution fluid is only guaranteed if the machine feed water falls within certain criteria (Penne et al., 2009). However, as ultrafilters are exposed to disinfection agents and cleaning cycles their efficacy in removing endotoxin is reduced. A desirable application would be a novel surface treatment or coating that would enhance endotoxin removal, while able to resist efficiency loss induced by age and chemical exposure. Durable membranes have been studied, with some researchers looking at ceramic or alumina membranes. As these membranes do perform well in retaining endotoxin, their expense in manufacturing seems to limit their application, as well as some membranes have been shown to leach aluminum when cleaned with NaOH (Bender et al., 2000).

The goal of endotoxin removal is ultimately to reduce patient inflammation, as contaminated dialysate has been linked to the systemic micro-inflammatory state observed in many hemodialysis patients (Ouseph et al., 2007). Other studies have reported links between inflammation and cardiovascular disease (CVD) in hemodialysis patients (Merino et al., 2008; Wang et al., 2011; Kerr et al., 2007), while some have shown association between inflammation and nutritional status (Raj et al., 2009), implicating the breadth of influence caused by repeated endotoxin exposure. As more ESRD patients are treated with high-flux dialysis membranes, the opportunity increases for bacterial contaminants in dialysis fluid to cross the membrane into the blood and activate numerous cell types, releasing pro-inflammatory cytokines which heighten the inflammatory condition (Vanholder et al., 1992; Almeida et al., 2006). This is also the case in the acute market, as membranes with increased permeabilities are being used in ARF for their higher clearances of cytokines (Haase et al., 2007; Vanholder et al., 2000); these patients will have higher risks for endotoxin contamination, based upon the membrane flux.

Aside from removal, advances in the detection of endotoxin and other pyrogenic substances at low concentrations would further propel endotoxin research forward by providing researchers tools to better distinguish bacterial contaminant changes, or by making highly sensitive endotoxin detection available to clinics to provide advanced microbiological observation of their water systems. Specific research in this field has focused on the efficacy

of utilizing photometry to detect and measure endotoxin in patient plasma samples (Nakazawa et al., 2010), which would have direct impact on research clinics and for septic patients. PCR techniques have also been utilized for their application to dialysis research, by analyzing total flora in dialysis water via the 16s rDNA. As this technique guarantees a high degree of detection, it does not specify whether bacteria or live or dead, or what species are prevalent within the sample (Nystrand, 2006).

4. Future work

Studies involving endotoxin in hemodialysis have been going on for quite some time – covering how to prevent and remove biofilm from water distribution systems, how endotoxin interacts with the body, and how to increase removal efficiencies. Going forward, future work may involve identifying new bacterial contaminants that cause adverse patient reactions, but may not be identified by the LAL assay (Glorieux et al., 2009). There are a number of smaller bacterial components released during cell lysis, with bacterial DNA fragments recently receiving considerable attention in research studies (Handelman et al., 2009; Schindler et al., 2004). Some of these studies have shown a correlation between bDNA fragments present in patient blood, and higher levels of CRP and IL-6 (Bossola et al., 2009). Current limitations in endotoxin quantitative methodology, which influence how bacterial contaminant results and target values are interpreted (Ledebo, 2007b) will hopefully be improved upon and expanded to cover additional areas of focus in ESRD treatment.

The future of any therapy used to treat patients with ESRD needs to focus on the associated mortality and morbidity influencing factors. Whether ESRD therapy will focus on smaller, wearable devices (Gura et al., 2008; Ronco & Fecondini, 2007), strive for increases in home treatment (Moran, 2009), or devices utilizing living cells (Humes et al., 2006), the effect of endotoxin must be taken into account for each application – and to address the specific actions necessary to remove endotoxin thus ensuring patient safety. Future progress in endotoxin research will hopefully alleviate inflammation-related complications, and improve patient outcomes for all aspects of ESRD.

5. Conclusion

In conclusion, endotoxin contamination of fluid for dialysis therapy is an important aspect of patient safety and well-being. Regardless of the microbiological quality of the water coming into the hemodialysis machine, the dialysis membrane is the final barrier between potentially contaminated dialysis fluid, and the blood of the patient. We have examined how manipulation of specific fiber membrane parameters (geometric properties, materials, coatings, chemical modifications) can be utilized to improve the endotoxin retentive properties to limit trans-membrane flux, whether they contribute to adsorptive improvements, sieving improvements, or both. Membrane structure, surface chemistry, material, and surface coatings all have an impact on how endotoxin is filtered and adsorbed from solution. In addition to better understanding endotoxin-membrane interactions, studies of endotoxin removal by membrane modifications will result in better approaches to manufacture dialysis membranes that remove endotoxin from solution quickly and with improved efficiency.

As future hemodialysis membranes are designed to further improve upon convective removal of larger middle molecular solutes such as B2M, the opportunity for pyrogenic

materials to enter the blood stream via back filtration becomes greater. An improved comprehension of how endotoxin is removed using a fiber membrane can lead to new improvements and future product designs, by streamlining concepts from development to production.

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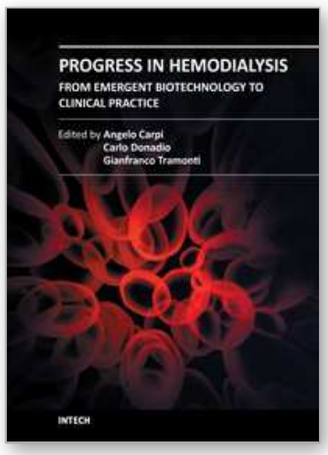

Progress in Hemodialysis - From Emergent Biotechnology to Clinical Practice

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Hemodialysis (HD) represents the first successful long-term substitutive therapy with an artificial organ for severe failure of a vital organ. Because HD was started many decades ago, a book on HD may not appear to be up-to-date. Indeed, HD covers many basic and clinical aspects and this book reflects the rapid expansion of new and controversial aspects either in the biotechnological or in the clinical field. This book revises new technologies and therapeutic options to improve dialysis treatment of uremic patients. This book consists of three parts: modeling, methods and technique, prognosis and complications.

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