New generation ceramic membranes have the potential of removing endotoxins from dialysis water and dialysate

Short Title: New ceramic membranes for endotoxin removal

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ABSTRACT

Poor water properties, use of concentrated bicarbonate, and biofilm growth in pipes and storage tanks often cause dialysis water and dialysate contamination with bacteria and endotoxins. High-flux dialysis with bicarbonate may favour endotoxin transfer from the dialysate into the blood exposing patients to serious short and long term side effects. Ultrafiltration across hydrophobic synthetic membranes effectively remove endotoxins from dialysis water by combined filtration and adsorption. However, repeated sterilization worsens the membrane separation properties, and limits their use. Ceramic membranes are generally more resistant to harsh operating conditions than polymeric membranes, and may represent an alternative for endotoxin removal. Previously, we proved that the ceramic membranes commercially available at that time were not retentive enough to ensure production of endotoxin-free dialysis water.

In this paper, we investigated the endotoxin removal capacity of new generation commercial ceramic membranes with nominal molecular weight cut-off down to 1,000. In dead-end filtration, all investigated membranes produced water meeting, or close to, the European standards when challenged with low endotoxin concentrations, but only one membrane type succeeded at high endotoxin concentrations. In cross-flow filtration, none produced water meeting the European standard. Moreover, sterilization and rinsing procedures altered the separation properties of two out of three membrane types.

KEYWORDS: Ceramic; Dialysis; Membrane; Sterilization; Water

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INTRODUCTION

At the beginning of the 90's, investigations in dialysis centers have revealed that a large fraction of the centers tested were using dialysis water and dialysate strongly contaminated with bacteria and endotoxins [1-4]. Klein et al. [1,2] reported that 53% of 51 centers investigated in the USA used dialysis water exhibiting bacterial count exceeding 200 CFU/ml. 35% of the centers used also dialysate with bacterial counts higher than 2000 CFU/ml. In 12% of the centers, endotoxin level in the dialysate was also above 5 EU/ml (i.e., endotoxin units per ml). In Germany, a similar survey in 30 dialysis centers revealed that 40% of the centers used dialysis water with bacterial count higher than 200 CFU/ml, and that 43% of the centers used dialysate with bacterial count exceeding 2000 CFU/ml. Endotoxin levels in dialysis water and dialysate were found to be higher than 5 EU/ml in 22% and 50% of all centers, respectively [3].

Microbial contamination of dialysis water and dialysate is acknowledged to be the cause of life-threatening acute and invalidating chronic side effects in patients undergoing hemodialysis [4]. To minimize the occurrence of such effects, national authorities have issued rules setting the maximum tolerable concentrations of bacteria and endotoxins in dialysis water and dialysate. The European Pharmacopoeia sets the maximal values of bacterial count and endotoxin concentration in the water used to prepare the dialysate at 100 CFU/ml and 0.25 EU/ml, respectively [5]. The American Association for the Advancement of Medical Instrumentation (AAMI) accepts higher bacterial counts in dialysis water with a limit of 200 CFU/ml [6]. However, the AAMI prescribes that bacterial count in the dialysate should not exceed 2000 CFU/ml, whereas the European Pharmacopoeia does not provide any limit for it. Neither authority sets limits for endotoxin concentration in the dialysate. In spite of experimental evidences, and the rules set by national authorities, dialysis centers are slowly including processes for endotoxin removal

in their water treatment plants on the assumption that the separation properties of dialysis membranes make them a safe enough barrier against endotoxins. It is now commonly agreed that endotoxins and their fragments do cross dialysis membranes irrespective of whether they are low or high flux [7]. Therefore, use of highly purified dialysate (i.e., concentration of bacteria < 100 CFU/ml, and endotoxins < 0.25 EU/ml) may be expected to improve the biocompatibility of the whole haemodialysis treatment.

The main contributors to microbial contamination have been proven to be the water used for preparing the dialysate, the bicarbonate concentrate, and the dead spaces in the fluid distribution system where a biofilm may develop [4]. Pre-treatment with filters, softeners, active carbon cartridges, microfilters, followed by treatment by reverse osmosis and/or de-ionization generally yields dialysis water meeting the official requirements. Prior to preparing the dialysate, water is generally filtered across sub-micron filters, and then treated with ultraviolet radiation [4]. The former remove whole bacteria but cannot clear endotoxins and their fragments. Ultraviolet radiations disinfect the water by killing bacteria but, by doing so, contaminate the water with bacterial wall fragments (i.e., endotoxins). Endotoxin removal from the dialysate is best achieved by on-line ultrafiltration through thick hydrophobic membranes (e.g., made of polysulphone or polyamide) that reject and adsorb large amounts of endotoxins [8-10]. Several studies clearly show the effectiveness of ultrafiltering the dialysate through endotoxin-adsorbing membranes just before it enters the hemodialyer [11,12]. However, the polymeric membranes used exhibit poor separation properties after repeated disinfection cycles, which limits the time they can be used. Ceramic membranes for ultra-/nanofiltration could be a convenient alternative to adsorptive filtration across polymeric membranes. In fact, they generally withstand the harsh conditions at which membranes are disinfected and heat sterilized better than polymeric membranes. Thus, they could be repeatedly used for quite a long time. In a previous

investigation, we showed that the ceramic nanofiltration membranes commercially available at that time did not effectively remove endotoxins from aqueous solutions [13,14].

In this paper, we report our investigation aimed at analyzing the endotoxin removal of new commercial ceramic membranes for ultra-/nanofiltration from aqueous solutions when they are operated either in cross-flow, or dead-end mode.

MATERIALS AND METHODS

We investigated three types of commercial tubular ceramic membranes for ultra- and nanofiltration, whose nominal properties are reported in Table 1. They were assembled in modules that were installed in the experimental apparatus shown in Figure 1, and were challenged with aqueous solutions spiked with endotoxins fed to the membrane lumen. Patent membranes were tested in the cross-flow mode, at 3 m/s tangential velocity, 25°C, 1.5 bar transmembrane pressure for 60 min. In an effort to simulate the worst possible conditions under which membranes might actually remove endotoxins, the test solutions were also filtered across the membranes in dead-end, single-pass mode for 40 min, at 25°C, 1.5 bar transmembrane pressure. Experiments where membranes were challenged with endotoxins for 5 h were performed after the membranes had undergone at least four complete sterilization and rinsing cycles. In each cycle, the membranes were sterilized at 180º degrees Celsius in a hot air sterilizer (SL600, Memmert, Schwabach, Germany), rinsed with 1 M NaOH and 60% ethanol, and rinsed again with highly purified pyrogen-free water [15]. The whole test equipment was subjected to the same rinsing procedure. Two membrane modules of each type were tested at given endotoxin concentration, and operating conditions. Change of the permeate flux at the given transmembrane pressure was used as an indicator of the occurrence of fouling, or of damages to the membrane selective "skin" layer.

Membranes were challenged with endotoxin-containing solutions at concentrations ranging from 0 to 2000 EU/ml. Endotoxins from *E. coli* were used throughout (*E. coli* Serotype 055 B5, Charles River, Kisslegg, Germany). Permeate samples were timely collected and assayed for endotoxins. Endotoxin concentration was measured with the kinetic turbidimetric Limulus Amoebocyte Lysate (LAL) Test (Charles River Endosafe, Kisslegg, Germany), with a micro titer plate reader (Sunrise, Tecan, Austria), and evaluated with the Endosafe software (Charles River Endosafe, Kisslegg, Germany). This method ensures sensitive detection of bacterial endotoxins, down to 0.125 EU/ml [16]. In this investigation, reference is made to the 0.25 EU/ml upper limit set for endotoxin concentration in dialysis water by the European Pharmacopoeia [5].

Membranes were generally characterized after their use. Membrane morphology was analyzed by scanning electron microscopy, after coating with gold under vacuum. Membrane pore size distribution was investigated by mercury intrusion with a AutoPore IV 9500 (Micromeritics Instrument Co., Norcross GA, USA) porosimeter. The membrane rejection coefficient spectrum was characterized by filtering a 0.8 g/l aqueous solution of polyethyleneglycols (PEGs) or Dextrans of different molecular weight (MW) (Sigma Aldrich, Steinheim, Germany) across the membranes in dead-end mode for 1.5 h at 20 l/m^2 h. Concentration of a given molecular weight solute in permeate, feed and retentate was estimated by GPC using a MZ Hema Bio column (MZ Analytik, Mainz, Germany) coupled to a refractive index detector (Waters, Milford MA, USA), with reference to PEG calibration standards. The rejection coefficient was estimated as $R = 1 - (2\ C_{permeate} / (C_{retentate} + C_{feed}))$. The molecular weight cut-off was estimated as the molecular weight of a solute rejected by the membrane to an extent equal to 90%.

RESULTS

Figures 2 a-f show that all investigated membranes exhibit a rather thick and asymmetric wall with an inner skin layer, supported by a porous layer of granular ceramics. The region in the supporting layer closer to the skin is generally made of granules of smaller size and has a lower porosity than that farther away. In membranes type #3, the granules in the supporting layer is flaky and less orderly distributed than that in the other membranes generally causing a lower porosity and the occasional formation of larger-than-average pores as shown in Figures 2 d-f. Mercury intrusion evidenced only the presence of pores in the range from 10 to 0.1 microns, presumably in the supporting layers. In fact, the thicker walls and the smaller porosity than those of organic membranes might make it difficult for intrusion techniques to detect pores in the skin layer of ceramic membranes.

Table 2 shows the endotoxin concentrations detected in the permeate of different patent ceramic membranes after 40-60 min from spiking water with the endotoxin bolus. When membranes #1 and #2 were operated in the cross-flow filtration mode and challenged with 1000 EU/ml endotoxins, neither of them produced water meeting the 0.25 EU/ml limit. In fact, endotoxin concentration in the permeated water was consistently higher than 0.5 EU/ml although lower than 5 EU/ml. Table 2 shows that, when operated in dead-end mode and challenged with low endotoxin concentrations, type #2 and #3 membranes yielded permeate water containing approximately 0.25 EU/ml endotoxins. Only type #1 membranes produced water meeting the European standards. When the endotoxin challenge was increased by an order of magnitude, only the most permeable membranes type #1 unexpectedly produced water with by far less than 0.25 EU/ml endotoxins.

After at least 4 complete sterilization and rinsing cycles, membranes type #2 and #3 exhibited altered water permeability. In fact, the permeate flux through type #2 membranes decreased to 280 ml/(min m^2) from the 1330 ml/(min m^2) value for the patent membranes.

The permeate flux through membranes type #3 dramatically increased to 5270 ml/(min m^2) from the 947 ml/(min m^2) value for the patent membranes. Type #1 membranes consistently yielded a permeate flux of 9500 ml/(min m^2). After 1.5 h dead-end filtration of a 100 EU/ml endotoxin challenge, all these membranes yielded permeate water with less than 0.25 EU/ml. It is noteworthy that membranes type #1 produced water with less than 0.05 EU/ml endotoxin. After 5 h dead-end filtration, endotoxin concentration in the permeate of membranes type #2 and #3 was less than 1.5 EU/ml and ca. 0.5 EU/ml respectively, and was less than 0.05 EU/ml in the permeate of membranes type #1. When challenged with 1000 EU/ml endotoxins, none of the tested membranes produced water meeting the 0.25 EU/ml requirement. In particular, endotoxin concentration in the permeate of membranes type #2 and #3 exceeded 5 EU/ml, and was generally less than 0.5 EU/ml in the permeate of membranes type #1. The tested membranes generally exhibited a rather slanted rejection spectrum after sterilization. Figure 3 shows that the membranes type #3 exhibited also a 19.500 Da molecular weight cut-off, largely exceeding their nominal 1.000 Da value.

DISCUSSION AND CONCLUSIONS

Endotoxins are cell wall components of gram-negative bacteria whose molecular weight is reported to range from 2000 to more than 100,000 [4]. The nominal molecular weight cut-off of the membranes used in this investigation (see Table 1) suggests that filtration of endotoxin-containing water across these membranes would yield sterile and endotoxin-free water meeting the strict European standards. Our results show that, when challenged with 100 EU/ml endotoxin in dead-end mode, the tested patent membranes produced water close to the 0.25 EU/ml requirement in the permeate. In particular, the membranes type #1 consistently produced permeated water with less than 0.05 EU/ml also

when challenged with high endotoxin concentrations. Comparison with the performance of the membranes tested in our previous investigations [13,14] clearly shows the great improvement in the manufacturing techniques of commercial ceramic membranes now delivering the expected performance *in vitro*.

The poor endotoxin retention of patent membranes type #1 and #2 when operated in cross-flow filtration mode suggests that the accumulation of rejected endotoxins upstream from the membrane plays an important role in determining the actual membrane rejection towards endotoxins. In fact, in dead-end filtration mode, endotoxins would concentrate at the membrane wall and might get trapped in the membrane pores as an effect of the poor solute back transport. At low endotoxin concentrations in the bulk, the trapped endotoxins would hinder permeation of the free molecules resulting in increased membrane rejection towards endotoxins. At high endotoxin concentrations in the bulk, the accumulated endotoxin overload would cause concentration polarization that decreases the observed membrane rejection towards endotoxins, as it was observed experimentally. Any enhancement of endotoxin back transport would decrease membrane rejection and would increase endotoxin concentration in the permeate, as it was observed in the cross-flow filtration experiments. Endotoxin adsorption at the membrane pore surface may be also evoked to explain the good endotoxin rejection of membranes type #1 in spite of their high permeability and large mean pore size. In fact, Figures 2d-f suggest that these membranes have a rather high specific contact area at least when compared to membranes type #3. Lack of information on the actual properties of the ceramics used prevents speculation on the existence of specific chemical interactions between endotoxins and membrane material.

The inconsistent performance of membranes type #2 and #3 before and after they underwent at least four complete sterilization and rinsing cycles brings up an unexpected limit to the tested membranes. In fact, the significant change of membrane permeability to

water suggests that repeated sterilization and rinsing might have seriously damaged or altered the membrane separation layer. Figure 3 confirms that this is indeed the case for the type #3 membranes whose molecular weight cut-off increased ca. twenty fold over the nominal value, consistent to the change of water permeation flux. Good news is that at least membranes type #1 successfully withstood repeated cleaning and sterilization procedures and consistently produced permeated water of good purity.

We conclude that some commercially available ultra-/nanofiltration ceramic membranes have the potential of removing endotoxins from dialysis water. However, poor endotoxin rejection in cross-flow filtration and in long-term dead-end mode hinders their use in routine hemodialysis at this time. Better understanding of the mechanisms leading to endotoxin removal, further improvement in membrane actual rejection properties and chemical stability should still be pursued to exploit some of their advantageous characteristics in the near future.

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Membrane Identity	Membrane #1	Membrane #2	Membrane #3
Geometry	Single channel	Single channel	Multiple channels
Number of channels	1	1	3
Material	$Al_2O_3/TiO_2/ZrO_2$	$Al_2O_3/TiO_2/ZrO_2$	$Al_2O_3/TiO_2/ZrO_2$
Length	250 mm	250 mm	250 mm
Nominal MW Cut-Off, NMWCO	20 nm[1,2]	5 kD[1]	1 kD[1]

Table 1. Properties of the ceramic membranes used in this investigation: [1] as indicated by the manufacturer; [2] average pore size

Operating mode	**Endotoxin challenge EU/ml**	**Membrane #1 20 nm**	**Membrane #2 5 kD**	**Membrane #3 1 kD**
Cross-flow Filtration	0	0*	0*	NA
	1000	<5*	<5*	NA
Dead-end Filtration	0	0	0	0
	100	<0,05	≤0,25	≤0,25
	1000	<0,05	<0,5	NA
	2000	NA	NA	<0,5

Table 2. Endotoxin concentration, EU/ml, in the permeate water across patent ceramic membranes after 40-60* min from the endotoxin challenge: NA: not available.

Figure Captions

Figure 1. Schematic of the experimental apparatus: BPV - back pressure valve; DV - discharge valve; M - manometers; MM - test membrane module; MU - muffler; P - pump; PD - pulse dampener; R - reservoir; T - thermometer; V – valve.

Figure 2. SEMs of the the investigated membranes.
Cross-section of: a - membranes type #1; b – membranes type #2; c – membranes type #3.
Magnification of the wall of : d - membranes type #1; e – membranes type #2; f – membranes type #3.

Figure 3. Rejection coefficient vs. molecular weight curve for type #3 membranes after sterilization. Membranes tested with an aqueous solution of PEGs of different molecular weight. See Materials and Methods for details.