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Development of amino-functionalized membranes for removal of microorganism

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Abstract

Treatments to ensure water supply of an acceptable hygienic-sanitary quality is of vast importance. Among unconventional treatments, membrane technologies have recently stood out. Immobilization of antimicrobial compounds onto membranes can prevent fouling and lead to self-cleaning matrices. In this study, cellulose membranes functionalized with amines were developed to assess their capability to remove microbial contamination. Water samples with several levels of *Escherichia coli* inoculum were filtered through membranes, and different trials were run to check the system's effectiveness. The amino-functionalized membranes were able to filter water samples in a few seconds, and partially or completely remove the inoculated microorganism depending on the inoculum level. The amine-functionalized membranes displayed significant retention capacity in samples with high bacterial concentrations and were able to decontaminate water with low microbial load. Membranes can be reused with no apparent loss of efficiency. Hence, this study demonstrates the high potential of amine-functionalized membranes in drinking water treatments.

Industrial relevance

Filtration represents an important non-thermal process used for clarification, concentration and microbial stabilization of liquid fluids. However, membrane fouling and related cleaning requirements are critical factors determining the extensive application of this technology. This work represents an important starting point to the development of new antimicrobial surfaces due to the demonstrated advantages associated to the covalent immobilization for the development of novel filtration treatment methodologies.

Keywords: *Escherichia coli*, filtration, immobilization, cellulose, polyamines, disinfection

1. INTRODUCTION

Water is essential for all living beings, which makes ensuring its safety in the moment when consumed a priority. Drinking water is defined as the water used for domestic purposes, such as drinking, cooking and personal hygiene, and is considered safe if it meets certain microbiological and chemical standards (World Health Organization, 2017). Unfortunately, the world's entire population cannot access necessary water supplies, and developing countries are particularly suffering serious water shortage consequences. Every year, millions of people suffer from different illnesses, such as cholera, diarrhea, dysentery, typhoid fever, etc., caused by drinking contaminated water. In fact it has been calculated that more than 502,000 people die per year due to diarrhea caused by drinking unsanitary water according to the WHO (World Health Organization, 2011).

Drinking water can be contaminated by chemical compounds, physical elements and biological species, such as viruses and bacteria, which pose a direct human health risk (Kroll et al., 2012). Pathogens derived from faeces are the main concerns for establishing health-based objectives for microbial safety. Thus, the WHO (2017) has set the absence of fecal coliforms, specifically *Escherichia coli*, *Enterococcus* and *Clostridium perfringens*; per 100 mL of water as a microbiological limit.

The growing demand of drinking water is today a worldwide challenge (Tiwari, Tiwari, Behari, & Sen, 2008). Therefore, running appropriate treatments to ensure that water supplies offer an acceptable hygienic-sanitary quality is of much importance. Approaches for ensuring microbial safety of drinking water are based on methods to prevent the contamination of drinking water or to reduce contamination to levels not adverse to health. The use of chlorine for water disinfection has been considered one of the best achievements of the 20th century in the public health field (Center for Disease

Control and Prevention, 1999). However, toxic or carcinogenic disinfection by-products (DBPs) are formed from using chemical disinfectants, such as chlorine, chloramine and ozone (Richardson, Plewa, Wagner, Schoeny, & DeMarini, 2007). The specifically use of chlorine may lead toxic compounds to form, such as trihalomethanes or chlorinated phenols, among others. Furthermore, the resistance of some pathogens to traditional chemical disinfectants renders it necessary to use extremely high doses, which means high DBPs levels in drinking water (Li et al., 2008). Hence, the pressing need to review conventional disinfection methods and to consider innovative approaches to both improve the disinfection reliability and avoid DBPs formation.

Different water treatment methodologies have been proposed to ensure water quality without affecting consumers health to prevent water shortage problems (Li et al., 2008). Membrane technologies stand out among the alternative water treatment systems available to treat wastewater, drinking water and water production (Salehi, 2014). Membranes allow the bacteria cells to be retained and provide purified water free of bacterial contaminants and DBPs (Kroll et al., 2012). This technology specially highlights in household water treatment (HWT) or point-of-use water technologies to treat collected water or contaminated piped water in developing countries. According to the guidelines for drinking-water quality stated by the WHO, the bacterial reduction achieved by membrane filtration in household water treatment technologies falls within the range of 1-2 or 2-4 $\log_{10}$ reduction value (LRV), for fiber and fabric filters and microfiltration, respectively (World Health Organization, 2011). However, membrane fouling is the most critical problem in membrane technology given the presence of biomolecules or microorganisms in water samples that hinder the water flux.

Immobilization of bioactive compounds onto membrane surfaces can prevent fouling and lead to self-cleaning matrices (Gialleli, Bekatorou, Kanellaki, Nigam, & Koutinas,

2016; Kroll et al., 2012). The use of functionalized surfaces by anchoring active inorganic compounds, e.g. silver (Dankovich & Gray, 2011; Gopiraman et al., 2016; Lala et al., 2007; Oyanedel-Craver & Smith, 2008; Sinclair, Zieba, Irusta, Sebastián, & Arruebo, 2014; Tankhiwale & Bajpai, 2009) or graphene (Hu et al., 2010), and active organic compounds, e.g. essentials oils (Royo, Fernández-Pan, & Maté, 2010), peptides (Nakamura et al., 2011), biocides (Mansur-Azzam, Woo, Eisenberg, & van de Ven, 2013) and enzymes (Kroll et al., 2012) has been recently proposed. This methodology provides simple, portable and cheap disinfection systems that do not require special equipment and efficiently reduce microbial density (Dankovich & Gray, 2011; Mansur-Azzam et al., 2013). There is no doubt that antimicrobial membranes are better than other traditional techniques for water treatment, but more studies still need to be done to assess the environmental impact of immobilized compounds (Tiwari et al., 2008). An alternative to the aforementioned systems could be the immobilization onto membrane surfaces of organic compounds, such as amines, which form part of some of the most important biological compounds that act as bioregulators, neurotransmitters, in defensive mechanisms and many other functions in living beings (Igarashi & Kashiwagi, 2010). Some polyamines, such as quaternary amines, have been widely reported as antimicrobial agents. Quaternary ammonium compounds with N-alkyl chains present antimicrobial activity given the association between positively charged quaternary nitrogen and negatively charged head groups of acidic phospholipids in bacterial membranes, which disrupts membrane integrity (Buffet-Bataillon, Tattevin, Bonnaure-Mallet, & Jolivet-Gougeon, 2012). Quaternary amines have been successfully anchored to membranes to create antimicrobial supports (Andresen et al., 2007; Kim, Nam, Park, & Park, 2007; Roy, Knapp, Guthrie, & Perrier, 2008). However, the application of such amines has been related to some toxicological issues and microbial

resistance (Aase, Sundheim, Langsrud, & Rørvik, 2000; Thorsteinsson et al., 2003). Otherwise, primary and secondary amines are not considered effective antimicrobial agents. Bartels et al. (2016) developed amino-silanized yttria stabilized zirconia capillary membranes for controlled virus retention based on the positive surface charge of the amino groups immobilized on the membranes for the adsorption of the negatively charged viruses. Following a similar approach in this study, we evaluated the development of cellulose membranes functionalized with a primary amine and assessed their capability to remove *Escherichia coli* in water samples.

2. MATERIALS AND METHODS

2.1. Chemicals

Standard paper grade cellulose paper (75 g/m^2) (product number RM13054252) was purchased from Labbox (Barcelona, Spain). *N*-(3-trimethoxysilylpropyl)diethylenetriamine (N3) (product number 413348) and dimethyl sulfoxide (DMSO) (product number 1029522511) were provided by Sigma-Aldrich (Madrid, Spain).

2.2. Preparing functionalized membranes

The surface of cellulose membranes was covalently functionalized with polyamines to create antimicrobial membranes for water sterilization. For that purpose, polyamine *N*-(3-trimethoxysilylpropyl)diethylenetriamine was covalently anchored to paper membranes. In a typical experiment, a solution of N3 (50% v/v) in dimethyl sulfoxide was prepared (Akhlaghi et al., 2015). Then, 1.5 mL of the mixture were added drop by drop to the paper membranes (⌀ 4.5 cm). Once membranes were well impregnated, they were dried at room temperature for 2 h. Finally, membranes were washed with distilled water (100 mL) to remove excess polyamines and were dried at 40°C for 24 h.

2.3. Membrane characterization

Paper membranes were characterized following standard instrumental techniques. A morphological analysis of the cellulose microstructure was performed by Field Emission Scanning Electron Microscopy (FESEM) observations. FESEM images were acquired with a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. The space between fibers (pore size) was estimated by averaging the measured size values of 50 replicates. Confirmation of membrane functionalization was determined by Fourier transform infrared spectroscopy (FT-IR), thermogravimetric analyses (TGA) and orange II assay. IR spectrums of membranes were obtained with a Bruker Infrared Spectroscopy Tensor 27 instrument (Massachusetts, US) between 4000 and 400 cm^{-1} in the transmittance mode. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland), using a heating program that consisted in a heating ramp of 10 °C/min from room temperature to 600ºC in a nitrogen atmosphere (50 mL/min). The quantification of the amount of accessible amino groups was determined by the orange II analysis based on the principle that one acid orange dye molecule binds to one amine group (Kroll et al., 2007). Amine-functionalized membranes were incubated in 1.75 mL solution of 0.5 mM acid orange II sodium salt in HCl (pH 3) for 24 h. After incubation, membranes were washed with abundant HCl solution (pH 3) to remove unbound dye molecules. The desorption of the bound orange II molecules was performed by pH-shift adding 2 mL NaOH solution (pH 12) followed by incubation for 15 min under shaking. The supernatants were spectrophotometrically analyzed at 483 nm using NaOH solution (pH 12) as blank sample and comparing the absorbance with that of NaOH solution with acid orange II at various concentrations.

The potential leaching of the immobilized aminosilanes was evaluated after washing of the membranes with 1 L of sterile water and then quantification of the accessible amino groups attached to the washed membranes by the orange II assay.

2.4. Microbiological assays

The bacterial strain used in the microbiological studies was a non-pathogenic strain of *Escherichia coli* K12 (CECT 433), obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). This strain was chosen for its role as a fecal contamination indicator in water according to the WHO (2017). Plate Count Agar (PCA) (product number 01-161-500) and Tryptone Soy Broth (TSB) (product number 02-200-500) were used to grow the microorganism. Peptone water (product number 02-568-500) and sterile distilled water were used to prepare decimal dilutions of the inoculum. Selective media Tryptone Bile X-Glucuronide (TBX) agar (product number 01-619-500) was used to plate the microorganism after treatment. All the media were provided by Scharlab (Barcelona, Spain).

2.4.1. INOCULUM AND INOCULATED WATER PREPARATION

The bacterial strain was reconstituted following the CECT instructions. Bacterial stock was stored at 4 ºC in PCA before use. The cells from an *E. coli* colony grown on PCA were transferred to 10 mL of TSB and were incubated at 37ºC for 24 h to obtain an inoculum with a density of approximately 1 x 10^9 cells/mL of broth. Different inoculum densities were tested from 10 to 10^8 CFU/mL of water. For this purpose, decimal dilutions were prepared in peptone water and subsequently, according to the studied inoculation density, the last dilution was prepared in sterile distilled water to obtain test tubes with 10 mL of contaminated water.

2.4.2. WATER FILTRATION ASSAYS

The filtration procedure was carried out using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) linked to a pump with a 6 L/min speed pressure/suction and connected to a conic polystyrene tube to collect the filtered sample.

Four different filtration tests were performed as follows:

Test 1: Study the retention capacity of the amine-functionalized membranes against five inoculum densities (10^4, 10^5, 10^6, 10^7 and 10^8 CFU/mL) by assuming the worst-case scenario to test the filtration efficacy of the developed membranes. In a typical experiment, 10 mL of inoculated distilled water (pH 6.10 ± 0.09) were filtered through a functionalized membrane and the permeate (>9.9 mL) was recovered in a conic polystyrene tube.

Test 2: Assess the efficacy of membranes by increasing the number of filtrates or the number of membranes. To this end, a distilled water sample with a microbial density of 10^4 or 10^6 CFU/mL was filtered 3 times with the same membrane or was filtered with three different membranes or three membranes together following the above-explained procedure.

Test 3: Study the reuse capability of the developed membranes. In this test, five different distilled water samples, with a microbial density of 10^4 or 10^6 CFU/mL, were filtered through the same membrane.

Test 4: The retention capacity of the amine-functionalized membranes was tested in distilled water samples using low microbial concentration having established the antimicrobial activity of the developed membranes against cultures in the stationary phase with a high microbial load. Regulatory agencies define a moderate risk of the

presence of >10 *E. coli* per 100 mL (Bain et al., 2014) and a very high risk exists when the number of coliforms per 100 mL is above 10^3 CFU (World Health Organization, 2011). Therefore, for this experiment 10 mL of water were inoculated with a bacterial density of 10 or 10^2 CFU/100 mL. Contaminated water was filtered through the amine-functionalized membrane and a sterilizing membrane filter (0.45 μm pore size) (EZ-Pak, Merck Millipore, Molsheim, France) that retained the microorganisms on its surface after the filtration procedure.

The same tests were carried out with the non-functionalized membranes used as a positive control. All the tests were performed in triplicate.

The antimicrobial activity of the filtration system was quantified by preparing serial dilutions of filtrates and plating them on selective media or by transferring the sterilizing filtration membranes to the culture media. Plates were incubated at 37 ºC for 24 h and viable cell numbers were determined as colony-forming units per mL (CFU/mL). These values were logarithmically transformed and expressed as log CFU/mL. Control positive values (water samples filtered through a non-functionalized cellulose membrane) were used to quantify the microbial count in the absence of treatment and to then calculate the percentage of bacterial reduction (in logarithmic basis) and the log reduction value (LRV).

2.5. Statistical analysis

Data were statistically analyzed with Statgraphics Centurion XVI (Statpoint. Technologies, Inc., Warrenton, VA, USA). The influence of different variables on bacterial viability was analyzed by analyses of variance (one-way and multifactor ANOVA). The LSD (least significant difference) procedure was used to test the differences between averages at the 5% significance level.

3. RESULTS AND DISCUSSION

3.1. Membrane characterization

The non-functionalized and amine-functionalized paper membranes were characterized using FESEM, Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analyses (TGA), as mentioned above. Figure 1 shows the microstructure of the non-functionalized (a, b) and the amine-functionalized (c, d) paper membranes obtained by FESEM. As observed in Figure 1a and c, the untreated paper membrane is formed by cellulose fibers that are more compact than the functionalized paper membrane. The amine-functionalized paper appears to have more separated cellulose fibers than non-modified membranes, which may be result from functionalization and the washing treatment. However, these differences are not observed in Fig. 1b and d, which show the fibrillary structure in more detail. According to FESEM analysis, control membranes present a mean pore size of ca. 38.9 ± 14.3 μm, whereas amine-functionalized membranes showed a pore size of ca. 41.4 ±.17.2 μm. In either case, the cellulose paper microstructure allowed microorganisms to pass through spaces of up to 10 μm between fibers at a reasonably fast gravity flow without resorting to pressure or suction (Dankovich & Gray, 2011), and also favored direct contact between bacteria and the immobilized polyamines. Fourier transform infrared spectroscopy (FT-IR) was carried out to confirm the attachment of polyamines to the cellulose paper surface qualitatively. Figure 2 shows the FT-IR spectra of the non-functionalized cellulose membrane and the amine-functionalized cellulose membrane. As we can see, the control cellulose membrane (upper line) presents a large band within 2000 to 2500 cm^{-1} wavelength range, which corresponds to the CH_2-OH bonds of the cellulose molecule. Low-intensity peaks appear at a wavelength above 3500 cm^{-1} due to the presence of absorbed

water being weakly bound that falls within 1600 to 1700 cm^{-1} range because of adsorbed water (Schwanninger, Rodrigues, Pereira, & Hinterstoisser, 2004). In contrast, the amine-functionalized cellulose membrane presents other representative bands. In this case, the band that corresponds to the CH_2-OH bonds and the peaks that corresponds to the presence of water are preserved, but new bands emerge, which confirms the immobilization of amines on the cellulose material surface. The presence of amines is observed on the peaks that correspond to N-H and NH_2 bonds, which fall within the 3000 to 3500 cm^{-1} range and the 1500 to1600 cm^{-1} range, respectively. The attachment of the trialkosysilane to the cellulose membrane is shown in the bands at 3700 cm^{-1} and 1200 cm^{-1}, which respectively correspond to the Si-OH and Si-CH_2 bonds, as reported in other studies (Hiyoshi, Yogo, & Yashima, 2005; Pacheco, Johnson, & Koros, 2012; Tumuluri, Isenberg, Tan, & Chuang, 2014).

Thermogravimetric analyses were carried out to confirm the immobilization of amines to the cellulose support. Table 1 shows the percentage of weight loss according to the different temperature ranges of the non-functionalized cellulose membrane and the amine-functionalized cellulose material. The non-modified cellulose membrane showed almost total degradation within the range of tested temperatures. Initially slight degradation was observed from room temperature to 100ºC, which corresponded to residual water. Major weight loss took place between 100ºC and 600ºC, which corresponded to 95.74% loss of organic matter, which means that the sample had almost entirely burned. This behavior is similar to that observed in previous studies (Yang, Yan, Chen, Ho, & Zheng C, 2007; Zhu, Sui, Wang, Sun, & Sun, 2004). In contrast, the N3-functionalized cellulose thermogram showed a different behavior and confirmed correct membrane functionalization. The initial mass loss (25-100ºC), which corresponded to water, represented a greater loss than for the control sample because of

the functionalization and washing processes. The second stage was similar to the non-functionalized membrane, but the percentage of weight loss was significantly lower in the third step. Total loss of matter was 70.27%, which is a much lower value than that of the control sample due to the presence of the trialkoxysilane group of immobilized amines, which degraded at higher temperatures. These results are in accordance with previous studies where the pyrolysis of amine ligands was complete by 700ºC (Zelenak, Halamova, Gaberova, Bloch, & Llewellyn, 2008), and the thermal decomposition residue of cellulose membranes after 600ºC was higher after the silylation with 3-aminopropyl trimethoxysilane by the increase of silicon associated to the alkoxysilane ligands (Saini, Belgacem, Salon, & Bras, 2016).

After confirmation of the attachment by qualitative methods, the accessible NH_2-groups/nm^2 on the amine-functionalized membranes were quantified by the acid orange II assay. The analysis of different modified membranes showed a mean amount of 10.11 ± 0.98 NH_2-groups/nm^2. The functionalization with N3 as an aminosilane with three amino groups per silane molecule resulted in a high amount of accessible NH_2-groups on the membranes' surface that can adsorb the microbial cells (Bartels et al., 2016). In addition, the potential leaching of the immobilized aminosilanes was evaluated by orange II assay after washing the membranes with 1 L of sterile water. The determination of the accessible amino groups attached on the membranes resulted in a mean amount of 10.86 ± 1.71 NH_2-groups/nm^2. Therefore, no wash-out effect after filtering high volume of water was observed, which confirms the covalent immobilization of the aminosilanes onto the membrane surface.

3.2. Bacterial retention assessment of the amine-functionalized membranes

The adsorption capacity of the amine-functionalized cellulose membranes was assessed against a high bacteria load (between 10^4 and 10^8 CFU/mL) by assuming the worst-case scenario to test the filtration efficacy of the developed membranes, as reported in other studies (Sinclair et al., 2014). Having established the inhibitory capability of membranes, the antimicrobial effect of the amine-functionalized cellulose membranes was evaluated in the water samples by simulating low microbial contamination with a bacterial density of 10 or 10^2 CFU/100 mL (Bain et al., 2014), as mentioned above.

The membrane flux of the non-functionalized and amine-functionalized cellulose membranes was in the same range (permeate volume >9.9 mL and filtration in less than 10 s), given the preservation of the membrane pore size after the immobilization process (Figure 1).

3.2.1. Retention activity of the amine-functionalized cellulose membranes according to inoculum density

Figure 3 shows the percentage of microbial reduction of the water samples inoculated with *E. coli*, with an initial inoculum density that range from 10^4 to 10^8 CFU/mL after filtration with an amine-functionalized cellulose membrane. The filtration of inoculated water through the non-functionalized membranes did not reduce the microbial load (data not shown). The developed membranes displayed a significantly different antimicrobial effect against *E. coli* depending on the inoculum density level ($p<0.05$), which was the biggest difference for the lowest bacterial load (10^4 CFU/mL). A reduction of 52±4% in logarithmic basis (equivalent to reduction of 99.96% of CFU) in the initial inoculum was achieved for inoculum density 10^7 CFU/mL. The maximum inhibition level was obtained for the highest inoculum density, obtaining a reduction of

3.74 ± 0.11 LRV. Although the maximum inhibition percentage did not exceed half of the initial microbial population, it is noteworthy that an inoculum was used in the stationary phase with a high microbial load, which did not simulate a real scenario. Furthermore, the inhibitory capability of the amine-functionalized membranes was remarkable if we take into account that the inoculated water filtration required only a few seconds.

The results obtained after single filtration of water contaminated with high microbial load through the amine-functionalized membranes meet the requirements stated by WHO for the point-of-use water technologies based on membrane, porous ceramic or composite filters. According to the guidelines for drinking water quality, filtration through fiber and fabric filters should reduce 1-2 LRV (World Health Organization, 2011), and these range was achieved by the amine-functionalized membranes for most of the inoculum densities (from 0.91 ± 0.05 to 3.74 ± 0.11 LRV, according to increasing inoculum level).

The bacterial retention capacity of the amine-functionalized membranes is related to the positively charged surface of the fibers due to the immobilization process. Despite zeta potential has not been analyzed in this study, previous authors have stated the conversion of the zeta-potential of the membrane surface from negative (non-functionalized) to positive values after amine-functionalization (Bartels et al., 2016). Due to the positive membrane surface charge of the amine-functionalized membranes, with high content of accessible amino moieties, the negatively charged bacteria (Habimana, Semião, & Casey, 2014) were adsorbed onto the membranes' surface through the filtration process.

The retention capability of a surface functionalized with N3 has been reported by immobilization of this polyamine on yttria-stabilized zirconia capillary membranes for

controlled virus retention (Bartels et al., 2016). Virus retention tests showed the substantial virus retention efficiency of the aminosilanized membranes after filtration of a viral solution through the capillary membranes. Amino-functionalized ceramic membranes displayed LRV levels of 9, being the result of the positive charge of the membranes that able to adsorb the virus because of the high content of accessible amino groups.

Other examples of the immobilization of bioactive compounds with antimicrobial activity onto cellulosic surfaces have been reported, despite they were not used as filtering elements. Saini et al. (2016) described the functionalization of cellulose nanofibers with other silane like 3-aminopropyl trimethoxysilane. The antimicrobial activity of the non-leaching antimicrobial surface was evaluated against different bacteria by zone inhibition test and quantitative antibacterial method, obtaining a reduction in bacterial concentration of 3.8 log for *E. coli* after 24 h of incubation of the inoculated films. A quaternary ammonium compound was grafted onto the surface of microfibrillated cellulose to develop non-leaching antimicrobial films. The antibacterial activity of the films was assessed by the zone inhibition test obtaining significant reduction in viable microorganisms (Andresen et al., 2007). Enzymes were anchored to modified cotton fabrics and their antimicrobial activity against bacteria and fungi was evaluated being able to inhibit the microorganisms even after consecutive wash cycles (Ibrahim, Gouda, El-shafei, & Abdel-Fatah, 2007).

3.2.2. Retention activity of the amine-functionalized cellulose membranes according to the number of filtrates or the number of membranes

After establishing the adsorption potential of the amine-functionalized membranes, the retention efficacy of membranes was evaluated after successive filtrations. To this end,

one water sample with an inoculum density of 10^4 or 10^6 CFU/mL was filtered 3 times with the same amine-functionalized membrane, and one sample was filtered 3 times with three amine-functionalized membranes or was filtered once using three membranes together.

Figure 4 presents the reduction percentage for *E. coli* after successive filtrations. The samples subjected to three filtrates obtained an inhibition average value of 31 ± 8 and 38 ± 3% for the 10^4 and 10^6 CFU/mL inoculum densities, respectively. The results were significantly different after three filtrations compared to the single filtration (Figure 3). The samples filtered 3 times with three amine-functionalized membranes presented a similar inhibition percentage to the three filtrates, for the 10^4 CFU/mL inoculum density, but the inhibitory effect was higher for that of 10^6 CFU/mL with a bacterial reduction of 51 ± 6%. The water samples subjected to filtration through three membranes together presented significant higher reduction (41 ± 7 and 46 ± 0% for the low and high inoculum density, respectively). These results are in accordance with the above-described test (Figure 3), where the higher the bacterial concentration, the stronger the retention effect of the developed membranes.

Similar to the results of Test 1, a reduction above 1 LRV was achieved after filtering the inoculated water (10^4 CFU/mL) through different membranes or after successive filtrations. For water inoculated with the high bacterial load (10^6 CFU/mL) the retention capacity resulted in 2-3 LRV, higher reduction than the values established in the guidelines given by the WHO for filtration technologies based on fiber and fabric filters (World Health Organization, 2011).

In spite of the slightly enhanced adsorption activity obtained in this test, it would be necessary to carry out studies with a growing number of filtrates to establish the

increase in antimicrobial activity after repeated filtrations (up to 50 times) as described in previous works (Sinclair et al., 2014).

3.2.3. Study of the reuse capability of the amine-functionalized membranes

The feature studied in this test was the reuse capability of the amine-functionalized membranes after filtering multiple samples. In a typical experiment, five water samples with a bacterial load of 10^4 or 10^6 CFU/mL were filtered through the same membrane, as explained in Section 2.4.2. Figure 5 shows the percentage of *E. coli* reduction after filtering the different samples. The retention of the bacteria population was boosted with a bigger number of filtrates, but samples did not significantly differ according to inoculum density or number of sample.

A certain growing trend in the inhibitory effect was observed with a larger number of filtered samples, which could be caused by a rise in the hydration grade of cellulose fibers after successive filtrates. Hydration could generate an increased fiber surface by reducing pore spacing, which could enhance the contact between samples and the polyamines attached to the cellulose membrane surface. The microstructure of cellulose fibers after hydration was studied by microscopic analysis, but this hypothesis could not be supported by the obtained results because the fibers of the membranes did not present significant differences in morphology and pore size (data not shown).

The reuse capability results indicated that the inhibitory effect was maintained while filtering the different samples because of the immobilization of the active molecule (polyamines) through a covalent bond. The results are in accordance with the quantification of the accessible amino groups results, which showed the preservation of accessible amino groups onto the membranes' surface after washing with high volume of water. This approach for obtainining membranes with antimicrobial properties, in

comparison with non-covalent binding methods, present the main advantage of the prevention of the release of the immobilized compounds, which can affect the properties of the treated matrix (Barbiroli et al., 2012).

3.2.4. Study of the retention capability of the amine-functionalized membranes on water samples with low microbial load

Finally, the adsorption activity of the amine-functionalized membranes was evaluated in the water samples by simulating the minimum microbial load that implies a risk for human health (Bain et al., 2014). To do this, samples were filtered through one amine-functionalized membrane 1 and 3 times, and were successively filtered through three membranes or were filtered once using three membranes together. A sterilizing membrane filter (mixed cellulose ester, 47 mm, 0.45 µm) was included at the bottom of the filtration system for microbial enumeration purposes, and also because its pore size allowed the microorganisms on the membrane surface to be retained after sample filtration.

Table 2 shows the microbial growth of *E. coli* in the water samples (inoculated with 10 and 10^2 CFU/100 mL) after filtering with the amine-functionalized membranes. Filtration through one membrane reduced both inoculum densities by around 40%. Filtering the water samples 3 times through one membrane enhanced the membranes' retention effect. The most remarkable results were obtained by successively filtering samples through three membranes or filtering them once using three membranes as an ensemble on the filtration device. The use of multiple membranes greatly removed the microorganism (a reduction around of 80-100%) after the filtration procedure. These results confirmed again the potential of the amine-functionalized membranes as filtering

elements due to their retention capacity (1-2 LRV) according to the guidelines for drinking water quality.

The adsorption capability of the amine-functionalized membranes is based on the electrostatic attraction that takes place between the activated positively charged membrane surface and the negatively charged microorganism. This binding, between the bacteria envelope and the accessible amino groups attached onto the surface of the cellulose membranes, might allow the attack of the cell wall with a local concentration of amines so high that it could easily disrupt the cell membrane and to induce the cell death (Huang, Wang, & Yan, 2010; Zhan et al., 2014).

In this work, the microbial retention capability of the developed membranes was performed with distilled water, where the only contaminant was the target bacterium. For drinking water treatment, a potentially significant disadvantage is membrane fouling by organic matter present in water that can be negatively charged and therefore able to adsorb to the positively charged membrane surface. Therefore, further studies with real water samples are necessary to validate the approach of bacterial removal.

4. CONCLUSIONS

In this work, a proof of concept of new antimicrobial membranes for water treatment was developed. Antimicrobial membranes were prepared through the covalent immobilization of polyamines on the surface of commercial cellulose paper. The amine-functionalized membranes showed excellent retention capacity in water samples with different inoculum densities for the Gram-negative microorganism *E. coli*. The results also demonstrated that the developed membranes can be used with either different filtrates or several membranes as an ensemble, and can be reused with no apparent loss of efficiency. The water contaminated with 10 CFU/100 mL of *E. coli* was completely

sterilized after performing high-speed filtration with the functionalized cellulose membranes. Therefore, the present study demonstrates the very high potential of the developed membranes in drinking water treatment thanks to several advantages, such as speed, easy handling, low cost and potential *in situ* use.

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Tables

Table 1. Percentage of weight loss of the non-functionalized and amine-functionalized cellulose membranes according to different temperature ranges.

Membrane type	25 – 100 ºC	100-300 ºC	300-600 ºC
Non-functionalized cellulose	1.75%	46.69%	47.30%
Amine-functionalized cellulose	4.97%	40.69%	24.61%

Table 2. Microbial growth of *E. coli* (log CFU/100 mL) in contaminated water after filtration with the amine-functionalized membranes (means and standard deviations, n=3).

	Inoculum concentration (log CFU/100 mL)	
Treatment	**10**	**10^2**
Control	0.82 ± 0.10^{aB}	1.72 ± 0.04^{aA}
1 membrane	0.48 ± 0.00^{bB}	1.11 ± 0.04^{bA}
3 filtrates	0.23 ± 0.40^{cA}	0.30 ± 0.00^{dA}
3 membranes	0.00 ± 0.00^{dB}	0.78 ± 0.15^{cA}
3 membranes together	0.00 ± 0.00^{dA}	0.15 ± 0.21^{eA}

Different superscripts denote differences ($p<0.01$) among count values from treatment conditions levels (small letters) and between inoculum densities (capital letters)

Figures

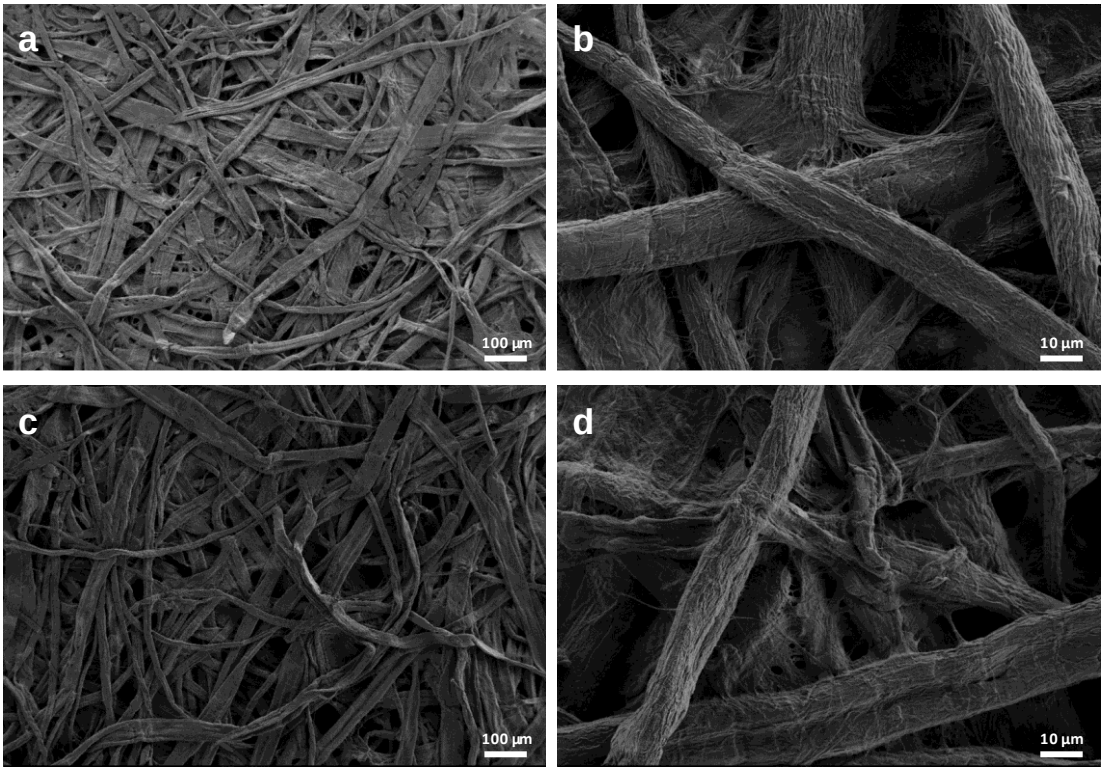

Figure 1. FESEM images of the microstructure of the non-functionalized cellulose membrane (a, b) and the cellulose membrane functionalized with *N*-(3-trimethoxysilylpropyl) diethylenetriamine (c, d).

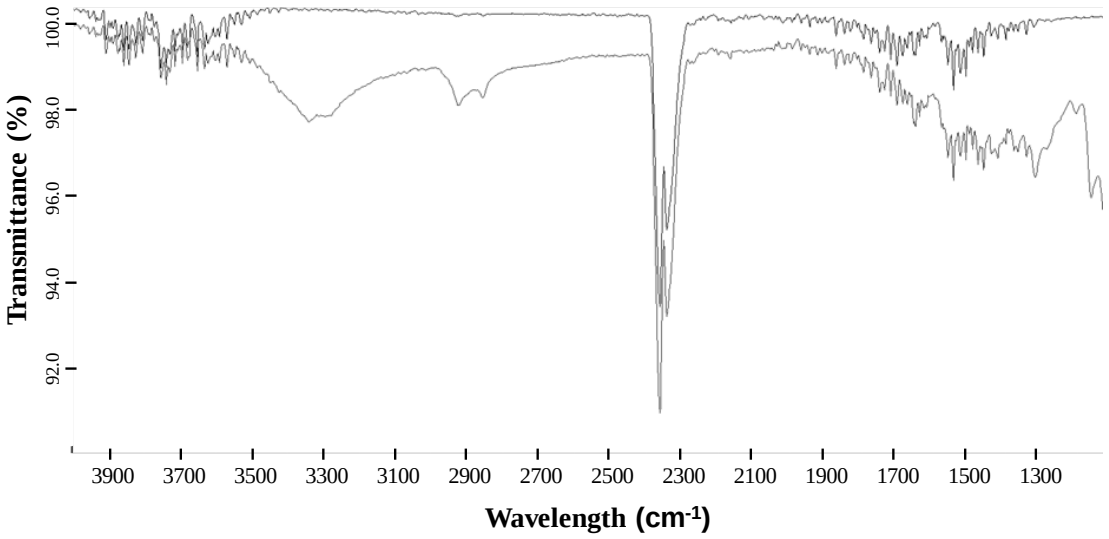

Figure 2. FT-IR spectra of the control cellulose membrane (upper line) and the cellulose membrane functionalized with *N*-(3-trimethoxysilylpropyl) diethylenetriamine (lower line).

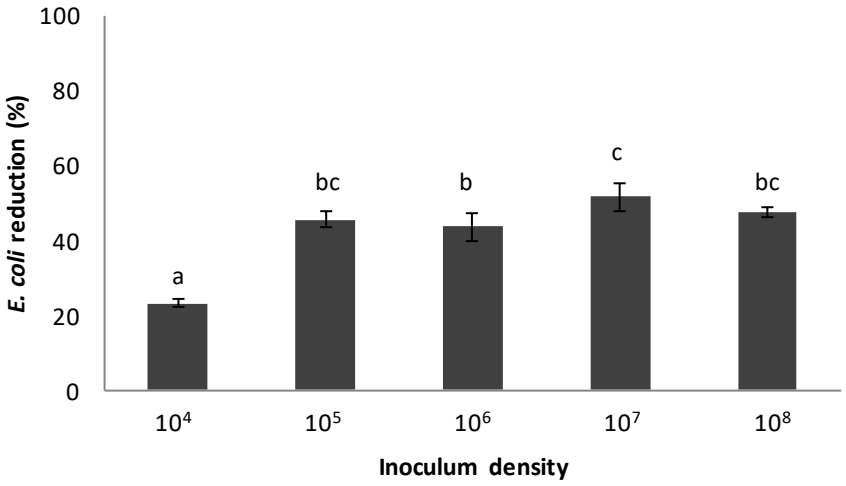

Figure 3. Percentage of reduced *E. coli* (after filtration with membranes with *N*-(3-trimethoxysilylpropyl) diethylenetriamine according to inoculum density. Different letters on the bars indicate significant differences ($p<0.05$) (means and standard deviations, n=3).

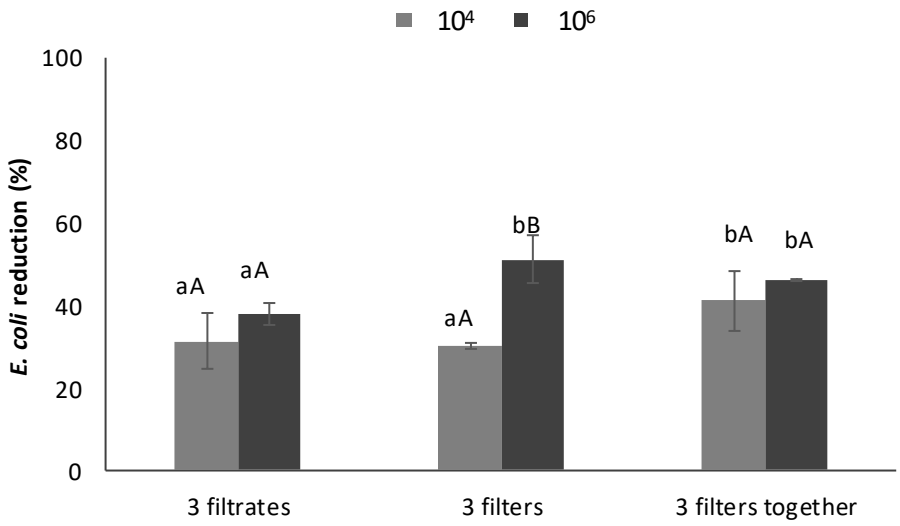

Figure 4. Percentage of reduced *E. coli* after filtration of one sample with one amine-functionalized membrane 3 times (3 filtrates) or after filtration of one sample with three amine-functionalized membranes (3 filters) or three membranes together (3 filters together). Different letters on the bars indicate significant differences ($p<0.05$) from the levels of inoculum densities (small letters) and the assay type (capital letters) (means and standard deviations, n=3).

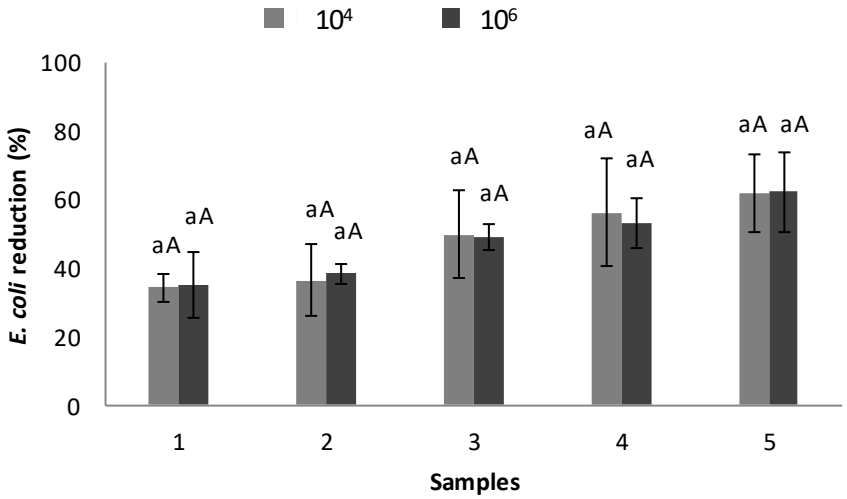

Figure 5. Percentage of reduced *E. coli* after filtration of five different samples with one amine-functionalized membrane. Different letters on the bars indicate significant differences ($p<0.05$) from the levels of inoculum densities (small letters) and the sample (capital letters) (means and standard deviations, n=3).