Anal Bioanal Chem (2018) 410:105–113
https://doi.org/10.1007/s00216-017-0695-x

RESEARCH PAPER

Development of an integrated method of concentration and immunodetection of bacteria

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Received: 21 June 2017 / Revised: 7 September 2017 / Accepted: 6 October 2017 / Published online: 23 October 2017

Abstract The microbial quality of water is a key aspect to avoid environmental and public health problems. The low pathogen concentration needed to produce a disease outbreak makes it essential to process large water volumes and use sensitive and specific methods such as immunoassays for its detection. In the present work, we describe the development of a device based on microfiltration membranes to integrate the concentration and the immunodetection of waterborne bacteria. A microfiltration membrane treatment protocol was designed to reduce the non-specific binding of antibodies, for which different blocking agents were tested. Thus, the proof of concept of the microbial detection system was also carried out using *Escherichia coli* as the bacterial pathogen model. *E. coli* suspensions were filtered through the membranes at 0.5 mL s^{-1}, and the *E. coli* concentration measurements were made by absorbance, at 620 nm, of the resultant product of the enzymatic reaction among the horseradish peroxidase (HRP) bonded to the antibody, and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB). The results showed that the homemade concentration system together with the developed membrane treatment protocol is able to detect *E. coli* cells with a limit of detection (LoD) of about 100 CFU in 100 mL.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-017-0695-x) contains supplementary material, which is available to authorized users.

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Keywords Microfiltration membrane · Sample concentration · Immunoassay · *E. coli* · Pathogen detection

Introduction

The detection of pathogens present in water is an increasing concern since waterborne pathogens are one of the leading causes of worldwide mortality. So, over the recent years, numerous epidemic outbreaks associated with waterborne pathogens have been the cause of about 5.7% of global diseases and 4% of deaths. In this context, the determination of microbial quality of the water has a great importance to avoid environmental and public health problems [1–3].

One of the biggest hindrances of waterborne pathogens is their low occurrence. As a consequence, large sample volumes need to be processed in order to increase the sensitivity of any methodology [4–8]. Preconcentration is the most common option to overcome this problem. There are several methods such as filtration, centrifugation, and immunomagnetic separation, which are used to reduce the sample volume and, therefore, increase the pathogen concentration enhancing its sensitivity [9]. Additionally, conventional methods such as colony counting, enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR) continue being the most commonly used [10–12]. Actually, the necessity of a rapid, sensitive, and inexpensive pathogen detection system still prevails. Moreover, to fulfill these characteristics, the developed system should be, as well, user friendly and portable, thus eliminating the transportation of the samples to the laboratory [13]. A potential way of addressing these objectives could be by using the microfiltration membranes to carry out the concentration processes as well as to support the immunologic reaction.

Microfiltration (MF) is an easy, direct, and simple way to reduce large sample volumes and concentrate the targets of

interest. MF is a size-based separation process in which a porous membrane acts as barrier retaining particles in the size range of 0.1–10 μm [14–16]. On the other hand, immunoassays are widely used bioanalytical methods, in which a biological target is detected due to its specific binding with a labeled antibody [6, 17]. However, the use of MF membranes acting as support of the immunoassays has also several drawbacks. The most important drawback to overcome is the non-specific binding of the antibodies to the membranes. It is well known that an important aspect that determines the specificity and sensitivity of a detection method is the signal-to-noise ratio [18]. For this reason, as it occurs with immunoassays, membranes need to be blocked to avoid unspecific antibody binding [19], the choice of the blocking agent being critical in order to decrease false positives.

In this paper, we present the development of an experimental protocol that enables the integration of bacterial concentration and detection processes using a unique substrate. The use of membranes allows us to process and concentrate larger sample volumes, while the developed protocol maximally reduces the non-specific binding of antibodies to MF membranes, improving the signal-to-noise ratio. The method was tested using *Escherichia coli* as model pathogen, obtaining an improved sensitivity with a faster detection and a lower cost than other methods.

Materials and methods

Membrane materials

Three different membranes with a nominal pore diameter of 0.2 μm were compared. These materials were nitrocellulose (NC) (Whatman Nitrocellulose, GE Health Care Life Science), polyvinylidene difluoride (PVDF) (Immuno-Blot PVDF Membrane for Protein Blotting, BioRad), and polycarbonate (PC) (Whatman Nuclepore Track-Etched Polycarbonate, GE Healthcare Life Science). *Escherichia coli* binding rabbit horseradish peroxidase-labeled polyclonal antibody (anti-*E. coli* antibody-HRP; E3500-06F, USBiologicals, Swampscott, MA, USA) with a final concentration of 2 μg mL^{-1} in PBS was used as antibody for all the experiments. As for blocking agents, proteins and surfactants were compared. As protein blocking agents, bovine serum albumin (BSA) (Sigma-Aldrich) and Western blocking reagent (WBR) (Roche Life Science) at concentrations of 0.5, 1, and 2.5% of the solution volume were analyzed. Tween-20 (Tw-20) (Sigma-Aldrich) and Triton 100 (TX-100) (Sigma-Aldrich) were checked as surfactants at concentrations of 0.1, 0.25, and 1%. All reagent concentrations were selected from molecular biology protocols where these blocking agents are commonly used [20, 21]. All blocking solutions were prepared using phosphate buffer 0.01 M (PBS) (Sigma-Aldrich) as solution base.

Membrane-blocking procedure

The different membranes (NC, PVDF, PC) were cut into 5-mm-diameter discs and treated with the blocking solutions (Fig. 1). For the blocking phase, the membrane discs were put in 500 μL of each blocking reagent (Tw-20, TX-100, WBR, BSA) and incubated for 2 h at 10 rpm using a rotator. In the case of the PVDF membranes, due to their high hydrophobicity, a pretreatment process was needed. For this reason, before the blocking step, PVDF discs were soaked in methanol for 5 min and washed in sterilized deionized water for 5 min.

Immunological reaction procedure

Blocked membrane discs (see section "Membrane-blocking procedure") were transferred to 500 μL of anti-*E. coli* antibody-HRP and incubated with the antibody for a period of 30 min in rotation at 10 rpm. After this, the membranes were washed to remove excess antibody. For this purpose, different concentrations of Tw-20 were tested (0.05, 0.1, and 0.5% in PBS). The membranes were washed three times in 500 μL of the washing solution for 5 min in rotation at 10 rpm. Finally, a last washing step was performed transferring each membrane to 500 μL of PBS (0.01 M) and incubating at 10 rpm for 5 min to remove the detergent of the membranes.

After the incubations and washing processes, the amount of antibody bound to the membranes was quantified by absorbance. The 5-mm-diameter membranes were placed on an ELISA 96 Microwell dish (Nunc-Immuno MicroWell 96-well plate, Sigma-Aldrich), and 100 μL of the colorimetric substrate Enhanced K-Blue (Neogen) was added to each well. This substrate contains 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2), and its reaction with the HRP conjugated with the antibody can be quantified by measuring absorbance at 620 nm using an ELISA reader (Multiskan EX, Thermo Scientific). The absorbance values were taken at minute 16, given enough time to observe a stabilization of the antibody-substrate reaction. Results were normalized by the corresponding blank, consisting of a membrane incubated without antibodies (Fig. 1), to compare the different blocking treatments. This resultant value was named normalized absorbance (AbsN).

$$\mathrm{AbsN} = \mathrm{Abs}(\mathrm{membrane} + \mathrm{Ab}) - \mathrm{Abs}(\mathrm{membrane}) \quad (1)$$

Likewise, the maximum non-specific binding (Fig. 1), the binding among the antibody and membranes without any type of blocking treatment, was also evaluated. All the performed measurements were carried out in triplicate, and averages and standard deviations were calculated.

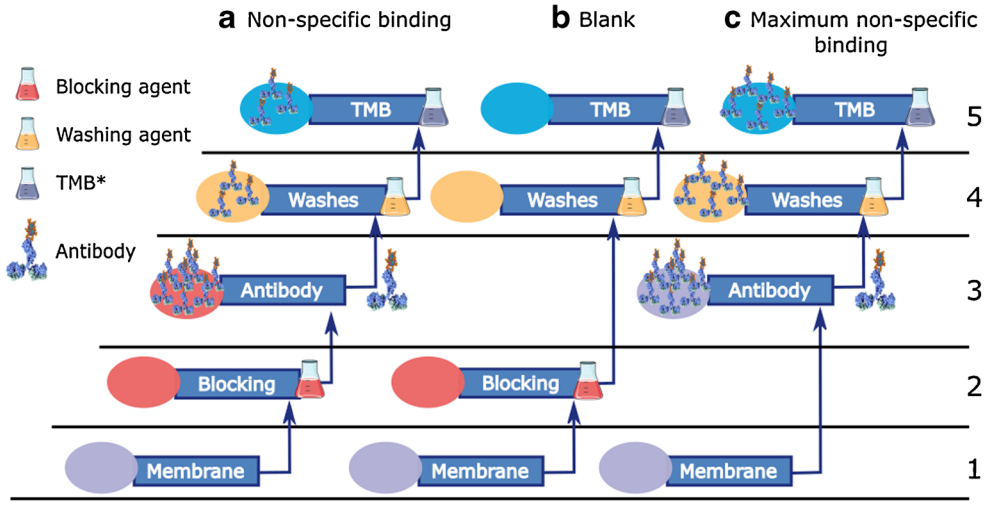

Fig. 1 Scheme of the immunoassay protocol developed on the microfiltration membranes. The numbers indicate each step of the immunoassay: non-treated membrane (1), blocking process (2), antibody incubation (3), washing (4), and TMB reaction. The columns indicate the different procedures carried out to the analysis of antibody-membrane reaction: **a** non-specific binding: treatment that includes the whole process, (blocking and anti-*E. coli* (HRP-Ab) incubation), **b** blank: blocking treatment without anti-*E. coli* antibody to obtain the intrinsic absorbance from each membrane material, and **c** maximum non-specific binding obtained incubating unblocked membranes with anti-*E. coli* antibody. (TMB*; 3,3′,5,5′-tetramethylbenzidine)

Custom-made concentration platform

The design of the concentration platform prototype employed for this work was carried out to integrate a sensing platform to the filtering procedure. The prototype, represented in Fig. 2, consists of a 42-mm-diameter cylinder, fabricated in polymethyl methacrylate (PMMA) divided in two parts. The upper part is formed by a reaction chamber of 20 mm in diameter and a groove of 3 mm in width to place the O-ring to ensure watertightness. The underside part has a planar surface provided with evacuation channels to facilitate water exit and reduce the pressure inside the holder. Additionally, both parts had threaded holes, to lock the holder and to adjust the standard Luer connectors (Plastic Value, threaded style Luer, polypropylene).

Bacterial cultures

Escherichia coli ATCC 10536 strain was grown at 37 °C overnight in 5 mL of Luria-Bertani medium (LB, Sigma-Aldrich). From this overnight culture, serial dilutions of *E. coli* were prepared in tubes containing 1 mL of Ringer solution (sodium chloride, Sigma-Aldrich) to obtain concentrations between 10^2 and 10^6 cells mL^{-1}. Finally, 100 mL of water was inoculated with the desired bacterial concentration. The concentration of the overnight culture and the prepared serial dilutions were estimated by plate count in LB agar. The colony-forming units per milliliter (cfu mL^{-1}) were calculated with the following formula:

$$\text{cfu mL}^{-1} = \text{number of colonies on plate}/(\text{dilution factor} \times \text{seeding } \textit{volume}) \quad (2)$$

Filtration processes

All the filtration processes were carried out by placing 25-mm-diameter membranes on the concentration platform and passing the sample through at a flow rate of 0.5 mL s^{-1}, employing a peristaltic pump (Gilson Miniplus3).

Testing of different blocking phases

In order to find out the best blocking procedure to enhance bacterial detection, the efficiency of different blocking procedures was analyzed. With this aim, water samples with a final *E. coli* concentration of 10^6 cfu in a volume of 100 mL were filtered through 25-mm NC and PC membranes blocked with Tw-20 at 1% or WBR at 2.5%.

Regarding the blocking phases, two different procedures were used. In procedure 1, the membranes were blocked for 2 h and, after this, bacterial samples were filtered. In procedure 2, the membranes were blocked for 2 h before and after sample filtration. In all cases, the membranes were blocked following the procedure described in section "Membrane-blocking procedure." A control was also carried out in which 100 mL of sterile distilled water was filtered.

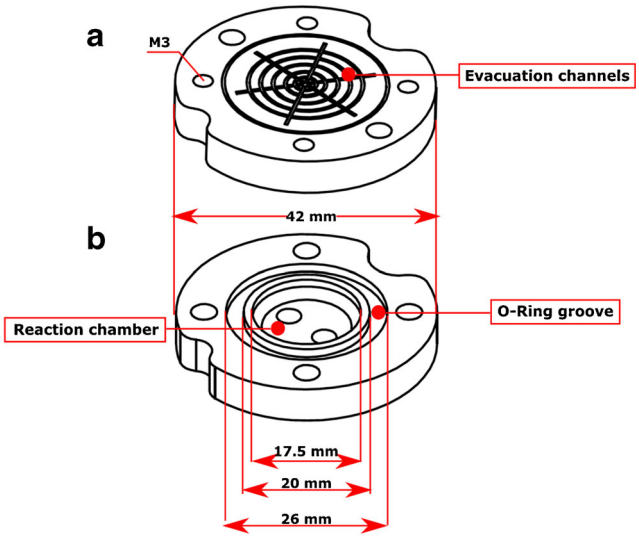

Fig. 2 Custom-made concentration platform. **a** Underside part, consisted of evacuation channels and metric 3 (M3) threaded wholes for the screws. **b** Upper part, provided with a reaction chamber for the different steps involved in the immunoassay and an O-ring groove to ensure watertightness. Threaded wholes are metric 3 (M3)

The membranes treated with each different blocking step were cut into four 5-mm-diameter discs as replicates. Three discs were transferred to tubes containing 500 μL of anti-*E. coli* antibody-HRP, and the fourth replicate was used to analyze the signal of the proper membranes (blank). Finally, the "Immunological reaction procedure" section was followed to perform the absorbance measurements.

E. coli detection and quantification calibration curve

In order to verify the capacity of the developed system to detect and quantify *E. coli*, we prepared samples with final concentrations between 10^2 and 10^6 total cells in 100 mL of sterile distilled water. The *E. coli* solutions were filtered through membranes blocked before and after filtration. Three whole membranes (25 mm) of each bacterial concentration as replicates were transferred to plates containing 2 mL of anti-*E. coli* antibody-HRP and incubated for a period of 30 min in agitation at 30 rpm. After this, the membranes were washed three times to remove excess antibody in 2 mL of Tw-20 at 0.5% for 5 min in rotation at 30 rpm. A last washing step was carried out with 2 mL of PBS (0.01 M) and incubating at 30 rpm for 5 min to remove the detergent of the membranes. Finally, the membranes were placed on a new plate and 500 μL of the colorimetric substrate Enhanced K-Blue was added to each well. After 16 min of reaction in agitation at 80 rpm, 100 μL of the resultant solution was transferred to an ELISA 96-microwell plate. Thus, the absorbance at 620 nm was measured by using an ELISA reader. Finally, the detection limit was determined by the following equation:

$$\text{limit of detection(LoD)} = \text{blank signal} + (3 \times \text{SD}) \quad (3)$$

Results and discussion

Effect of the blocking and washing solutions on the reduction of non-specific binding

Protein- and detergent-based solutions are the most employed blocking agents in assays that imply the use of membranes as support for immunoreaction processes such as immunoblots, ELISA, or enzyme-linked immunofiltration assay (ELIFA) [20, 22–27]. However, since a perfect and standard blocking agent does not exist, the selection of the best solution needs to be optimized for each method [20, 28]. In this way, two protein (BSA and WBR)- and two detergent (Tw-20 and TX-100)-based solutions were analyzed as blocking reagents at three different concentrations selected from several immunoassay protocols available in literature (0.5, 1, and 2.5% for proteins [20, 26] and 0.1, 0.25, and 1% for detergents [29–31]).

Normally, washing solutions are prepared with low detergent concentrations such as Tween-20 (Tw-20) to rinse excess antibody deposited on the membrane by breaking weak bonds among both [26, 32]. So, three concentrations of Tw-20 (0.05, 0.25, and 0.5%) were tested as washing solutions after incubating the membrane with the antibody. Tw-20 was chosen for this aim as it has been commonly used for these purposes in immunoassays [33–36]. The results were compared to the signal obtained with non-treated membranes, which shows the maximum non-specific binding.

The absorbance data obtained by the three membrane materials have been illustrated in Fig. 3. In all cases, the normalized absorbance (AbsN) (*Z*-axis) in relation to the washing (*X*-axis) and blocking (*Y*-axis) solution concentration is represented. All data obtained in these experiments are available in the Electronic Supplementary Material (Table S1).

Protein-based blocking solutions

In reference to BSA (Fig. 3(a)), a reduction of the non-specific binding was appreciated after the membrane treatment for all the materials. In general, the results indicated that the minimum BSA concentration used was enough to block the membranes.

In detail, PC (Fig. 3(a1), ESM Table S1) showed the best results with a non-specific binding reduction close to 100% for all concentrations and washing steps. In contrast, the results obtained by NC and PVDF membranes showed that non-specific binding reduction was related to the washing solution concentration (Fig. 3(a2, a3), ESM Table S1). In general, 0.5% concentration of Tw-20 was the best washing solution concentration reducing the unspecific binding about 78 and 89%, in the case of NC and PVDF, respectively, for all BSA concentrations (Fig. 3(a2, a3), ESM Table S1).

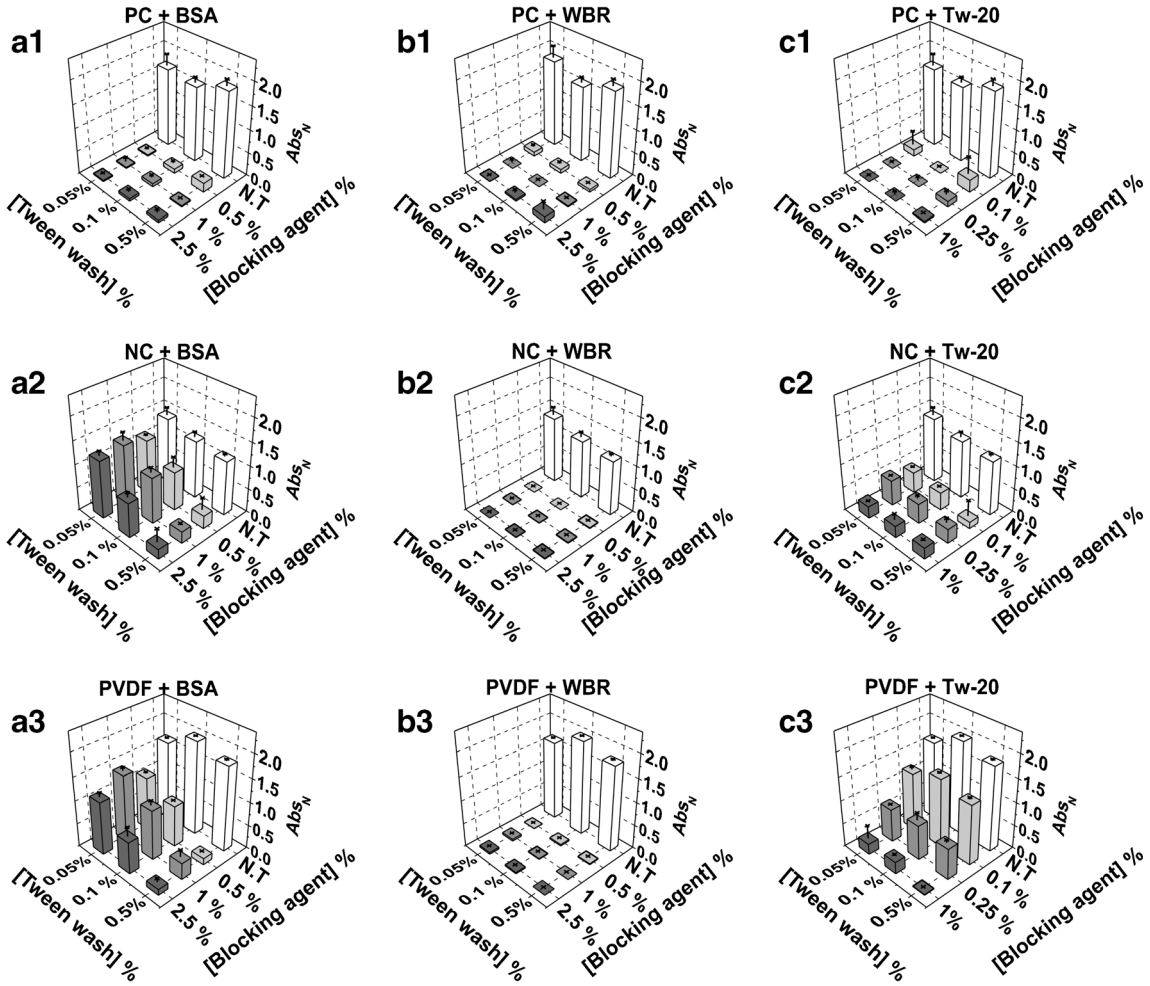

Fig. 3 Representation of the normalized absorbance (AbsN) measured for the different blocking treatments BSA (a), WBR (b), and Tw-20 (c) and the three membrane materials PC (1), NC (2), and PVDF (3). In all cases, the normalized absorbance (AbsN) is represented (*Z*-axis) in relation to the washing concentration (*X*-axis) and the blocking agent (*Y*-axis) solution concentration

In the case of WBR which is represented in Fig. 3(b), a great efficiency of this blocking agent was observed for the three membrane materials presenting the lowest non-specific binding (Fig. 3(b), ESM Table S1). As opposed to the case of BSA, washing steps were not necessary to improve this reduction.

Detergent-based blocking solutions

In Fig. 3(c), the results obtained for different membrane materials treated with Tw-20 are shown. In all cases, the reduction of the non-specific binding was related to the Tw-20 concentration. The best performance was obtained by the 1% concentration with a final reduction of the non-specific binding close to 100% for the PC membranes (Fig. 3(c1) and 90% for the NC and PVDF membranes (Fig. 3(c2, c3)).

In contrast, TX-100 was the most inefficient blocking reagent for the three materials. In fact, TX-100 had no effect on membrane/antibody binding reduction and all the measured values were equal (ESM, Table S1). Probably, TX-100 was not able to interact with the functional groups of the membranes and, as a consequence, the unspecific binding of antibodies was not reduced.

In general, the results showed that the highest concentration of all blocking agents led to the best results of unspecific binding reduction for all membrane materials. This indicates that the same blocking concentrations employed in immunoblot assays are necessary in this system since high concentrations of the blocking solutions are essential to avoid the unspecific binding membrane/antibody [20, 26, 30]. Regarding the differences observed between NC, PVDF, and PC, these could be related to their structures. NC (s. ESM, Fig. S1) and PVDF (s. ESM, Fig. S2) membranes are a complex mesh; therefore, the blocking solution could not be able to cover all the fibers of their structures. On the other hand, PC (s. ESM, Fig. S3) is a perforated flat polymer surface, which enables the formation of a uniform layer of blocking agent with better results.

Washing steps

The effect of the washing steps was also analyzed. The results showed that the higher unspecific binding reduction was obtained with the 0.5% concentration being selected for the following experiments. Some other authors also described that, despite lower concentrations being usually employed in immunoblotting protocols, a major decrease of this type of binding is obtained when 0.5% Tw-20 is used as washing solution [27, 37].

Comparison of blocking phases

In this study, we looked for the best blocking performance which could allow having bacterial detection with the objective of the future integration of the membrane-blocking protocol inside the reaction chamber of a simple device. In the light of the previous results, WBR at 2.5% and Tw-20 at 1% were selected as blocking agents and NC and PC as membrane materials. PVDF membranes were discarded for these experiments since their hydrophobic nature caused problems during water sample filtration. Additionally, *E. coli* was used as model of fecal bacteria since it has been used as a fecal contamination indicator since the nineteenth century [2, 11, 38–40].

Once the membranes had been blocked, prior to the sample filtration (Fig. 4a, c), we analyzed the signal differences obtained with clean (no *E. coli*) and bacterial (*E. coli*) samples in reference to the results obtained with non-treated membranes. On the one hand, NC membranes treated with WBR 2.5% showed low absorbance values when samples without *E. coli* were filtered (Abs = 0.075) with respect to non-treated membranes (Abs = 0.669). This indicated that WBR remained covering the membranes even after sample filtration, and therefore, a low non-specific binding of the antibody was observed. On the other hand, when Tw-20 was used as the blocking agent, the absorbance values obtained by the NC membranes treated (Abs = 0.745) and not treated (Abs = 0.669) were very similar. So, non-specific binding of the antibody to the membrane was not reduced, suggesting that blocking was lost during the filtration performance. This could be due to the fact that Tw-20 is a detergent and the passage of water could remove it from the membranes.

When samples containing *E. coli* were filtered, no increase in absorbance values was observed (Abs_{WBR} = 0.075, Abs_{Tw} = 0.592) independently of the type of blocking agent used. These could be attributed to the morphology of the membranes being of great importance for the detection system. NC membranes have a fluffy structure, formed by a complex mesh without straight pores with cavities and large surface areas [30, 41] (s. ESM, Fig. S1). For this reason, the pressure exerted by the water flow (30 mL min^{-1}) seemed to make the cells penetrate into their structure and not remain on the surface, thus hindering its detection.

On the other hand, the absorbance values measured for PC membranes treated with WBR and Tw-20 were higher than 1.4 and 1.6, respectively, even when the filtered samples were

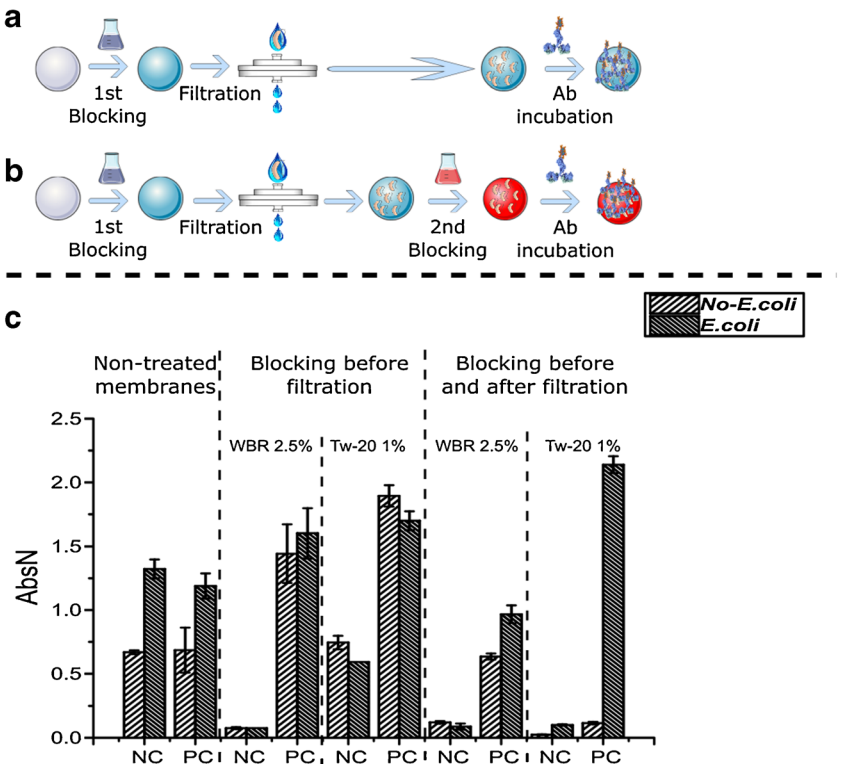

Fig. 4 Blocking phase comparison. **a** Scheme of the membrane blocking treatment before the filtration process. **b** Scheme of the membrane blocking treatment before and after the filtration process. **c** Normalized absorbance values obtained by membranes treated with WBR 2.5% and Tw-20 1% before and before and after filtration. Both cases are compared to the results obtained for the non-treated membranes

not inoculated with *E. coli*. PC membranes have flat surfaces with straight-through pores being ideal substrates for rapid microbiological test methods (Fig. S3), such as methods employing optical sensors [42, 43]. However, the fact that water samples need to be filtered through them to retain the bacteria caused the elimination of the blocking treatment and, as a result, the non-specific binding of the antibody to the membrane increased (Fig. 4c). In general, the results showed that the morphology of the membranes may have an important role and suggested that the water flow through the membranes affected the blocking performance.

Since the blocking step prior to the filtration resulted to be insufficient, a second post-filtration blocking step was tested (Fig. 4b). The results obtained for the membranes treated with the blocking agents before and after the sample filtration are shown in Fig. 4c.

In terms of the best material, the results showed that NC membranes blocked twice with WBR 2.5% or Tw-20 1% were not able to distinguish between the clean water sample (no *E. coli*) and the bacterial suspension (*E. coli*). This could be related again to the structure of NC. In contrast, for PC membranes, while the results obtained with WBR as blocking reagent did not show significant differences to the non-treated PC, the Tw-20 double blocking (before and after filtration) permitted having bacterial detection. As it can be observed in Fig. 4c, an absorbance value of 0.669 obtained with the blank (no *E. coli*) for the non-treated was reduced by about 83% employing Tw-20 as blocking agent. Besides, when membranes were treated with Tw-20, high signal differences (94%) were observed between samples with and without *E.coli*, 2.139 and 0.115 respectively. So, PC membranes blocked with Tw-20 showed a wider range of sensitivity to allow the distinction of different and lower bacterial concentrations and, therefore, they were selected as the best material and treatment.

E. coli detection and quantification of the calibration curve

Finally, a last experiment was carried out with PC membranes treated with the double-step blocking protocol, using Tw-20 at 1% as blocking agent, to test the capacity of the system to detect and quantify different concentrations of *E. coli*.

In Fig. 5, the relation between the absorbance and the different *E. coli* concentrations analyzed is shown. It could be appreciated that the higher the concentration of *E. coli* present in the sample, the higher the obtained absorbance value at 620 nm. Moreover, this calibration curve maintained a good linearity with an R^2 value of 0.99. Abdel-Hamid et al. (1999) developed an immunosensor based on nylon membrane filters able to obtain results in 30 min after filtering 1 mL at 0.12 mL min^{-1} [17], and Eltzovand Marks [44] developed a flow stacked immunoassay consisting of different

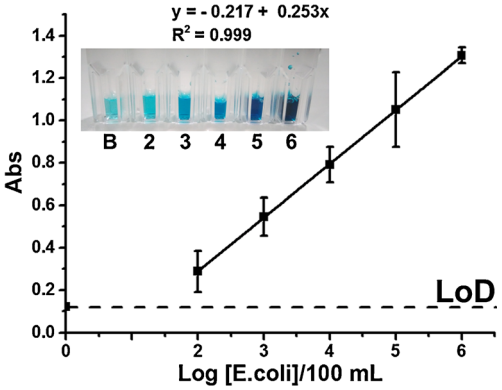

Fig. 5 Relationship between the absorbance measured at 620 nm (min 16 of reaction) and the *E. coli* concentration present in 100 mL water samples. The dashed line corresponds to the limit of detection (LoD) of the system

nitrocellulose pads with various components. Both methods obtained a threshold sensitivity of 10^2 cfu mL^{-1}. These methods succeeded in overcoming large assay times but continued working with low sample volumes. Dharmasiri et al. (2010) developed a microfluidic chip followed by quantitative PCR to cell enrichment and detection with a LoD of 6–10 cfu from 100-mL samples in 5 h. However, 100 mL needs to be filtered to reduce the volume to 1 mL before using the microfluidic chip [45]. In contrast, our system takes only a few hours (2–3 h), overcoming large assay times, and is able to handle larger sample volumes (100 mL), working at high flow rates (30 mL min^{-1}) without any preprocessing step. Additionally, the system presents a LoD of about 10^2 *E. coli* cells in 100 mL with a variability of 14%. Besides, the integration of sample concentration and immunoassay processes into a single device could endanger benefits such as a lower reactive consumption and an easier automatization.

Conclusions

In this research paper, we present a new waterborne pathogen concentration and detection system. With the aim of the integration of concentration and immunodetection processes into a single membrane, we designed a homemade filter holder to support a microfiltration membrane. Additionally, we developed a protocol to reduce the non-specific binding of antibody to microfiltration membranes allowing bacterial detection. This way, filtration through polycarbonate membranes blocked with Tween 20 at 1% before and after filtration processes, and washed with Tw-20 at 0.5%, was the most effective membrane treatment allowing *E. coli* detection and quantification, detecting about 10^2 *E. coli* cells in 100 mL of water (1 cell mL^{-1}). The obtained calibration curve clearly showed the detection capability of this protocol and confirmed that the developed detection system could be used for the determination of bacterial concentrations in water samples.

Additionally, future work is focused on fully integrating the protocol into the holder and automating the system in order to achieve a fast and simple bacterial detection device.

Funding information The authors acknowledge financial support from the Spanish Ministry of Economy and Competitiveness (BACSYS project CTQ2014-54553-C3-1-R). N.U. and O.C. acknowledge funding from the People Programme (Marie Curie Actions) of the 7th Framework Programme of the European Union (FP7/2007-2013), TECNIO-spring program from the Agency for Business Competitiveness of the Government of Catalonia (ACCIÓ). J.J.E. acknowledges financial support from the Catalan Industrial Doctorate program and Waterologies. S.L. and N.P. acknowledge financial support from Gas Natural Fenosa.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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