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Ten Million Fold Reduction of Live Bacteria by Bactericidal Filter Paper

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An extremely efficient bactericidal filter paper is developed that is capable of removing 99.99999% of *Escherichia coli* bacteria in a simple filtration process. The novel approach utilizes two active bactericidal components: a bactericidal agent, triclosan, which acts synergistically with a cationic polyelectrolyte binder with antibacterial properties. The biocide is incorporated into the block copolymer micelles attached to the cellulose fibers via the cationic polyelectrolyte. As the water containing the bacteria is passed by gravity through the filter paper, the bactericidal agents are transferred to the bacteria through collisions with the micelles or coated fibers. A synergy between the biocide and the polyelectrolyte is responsible for the extremely high efficiency in deactivating the bacteria. The filtered water is free of biocide other than that transported by the dead bacteria. This technology represents a very simple approach to provide potable water under a wide range of primitive conditions.

1. Introduction

The reduction of living pathogenic bacteria in water is important because of the obvious public health implications. It is especially relevant in countries which commonly lack safe drinking water, and in developed countries, in the case of a major catastrophe. Solutions to potable water shortages have been addressed through commercially available products such as drinking straws,[1] life saver bottle,[2,3] antimicrobial paper[4–7] or disinfecting pills.[8] Bactericidal paper based on impregnation with silver nanoparticles for point-of-use water treatment was previously described[9] The present method offers the simplicity of passing the water through antibacterial filter paper by gravity to achieve potability, while simultaneously removing particulates. It is simple and universally accessible, usable anywhere without any special equipment, easy to transport, easy to deploy, inexpensive, safely disposable, with an indefinite shelf life,[10] and most importantly, extremely efficient in bacteria reduction while producing water containing safe levels of the biocide.

The present approach involves the use of a hydrophobic biocide of extremely low water solubility, which is incorporated into the hydrophobic core of micelles made from amphiphilic block copolymers. The micelles (polystyrene-block-polyacrylic acid (PS-b-PAA)) are attached via a bactericidal cationic polyelectrolyte binder (CPE, specifically a cationic polyacrylamide (c-PAM) or poly(isopropanol dimethylammonuim) chloride (PIDMAC) to the filter paper fibers. Both these polymers find applications in papermaking and adsorb on cellulose fibers and thus can act as a "glue" to attach the block copolymer micelles to the fibers. Both polymers are commercially available. Their structures are given in the Supporting Information. Utilization involves simply passing the bacteria contaminated water through such a filter. The structure of the filter paper (treated Whatman filter papers of 6–8 μm pore size) allows the bacteria to absorb the biocide molecules via repeated collisional contacts both with the loaded micelles (from which the biocide is released), and the CPE treated fibers during flow; however, the attachment of the micelles and the polyelectrolyte to the fiber surfaces prevents the micellar carriers of the biocide molecules from entering the filtered water. Incorporating an antibacterial agent into amphiphilic copolymers has been reported before (silver nanoparticles in polypropylene-g-polyethylene glycol),[11] but these systems were not incorporated in filters. Also, these graft copolymers do not form micelles.

It should be stressed that the primary purification mechanism is not the removal of the bacteria from the water by filtration, but the deactivation of the bacteria as they pass through the filter paper. The reasonably rapid filtration can be accomplished by gravity alone without the need of pressure or suction. With time the deactivation efficiency of the filter is reduced, as biocide is transferred from the micelles to the bacteria, when bacteria pass through the filter. The highest efficiency of tested components of the filter is achieved by employing triclosan as the biocide and PIDMAC as the binder, and is higher than that of any other filter system since it reduces the bacteria count by 99.99999% in a single pass, i.e. a reduction by seven orders of magnitude.

Both the biocide (triclosan) and the CPE binder (PIDMAC) are the active components in killing the bacteria, and act synergistically to reduce the bacteria count. If the biocides were used alone, the efficiency in killing the bacteria and the bacteria reduction would not be as high (triclosan combined with cPAM as the binder ≈1 order of magnitude, PIDMAC alone ≈4-5 orders of magnitude); the combination of effects of both biocides acting synergistically on the bacteria is responsible for

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the extremely high efficiency (7 orders of magnitude). This is a novel approach in that it is based on synergistic action of two components.

2. Materials and Methods

2.1. Materials

Commercial Whatman filter papers of grades 2 and 3, of 110 and 90 mm diameters and of pore sizes of 8 and 6 μm, respectively[12] were used in this study. In order to achieve the antibacterial properties of the filter paper, the Whatman filter papers were first treated with the cationic polyelectrolyte binder and then with the biocide loaded micelles. The choice of biocide, loaded into block copolymer micelles of PS_{197}-b-PAA_{47}, was triclosan, a hydrophobic material of low water solubility.[13] The cationic polyelectrolytes were polyacrylamide (c-PAM) and PIDMAC. Gram negative *E. coli* bacteria (strain ATCC 11229) were used as a model pathogen; the overall net bacteria surface is negative.[14]

Detailed information about materials used in the present work, as well as their chemical structures and properties are given in Section 1 in the Supporting Information.

2.2. Incorporation of Biocide into Block Copolymer Micelles

The micelle preparation, starting from the amphiphilic PS_{197}-b-PAA_{47} block copolymer, and the characterization procedures of the micelles using transmission electron microscopy and dynamic light scattering were discussed in detail in previous papers.[15,16] Very brief descriptions are also given for the convenience of the reader in the Supporting Information.

The block copolymer micelles were loaded with the triclosan biocide and its concentration was evaluated by UV-vis, discussed in previous publications.[15,16] A brief description of the procedure is given in the Supporting Information.

2.3. Preparation of the Biocidal Filter Paper

The preparation of the bactericidal filter paper consists of four steps. Because the charge of the pulp fibers in the filter paper is negative, and loaded micelles with a negatively charged corona are to be attached, the ionic charge of the pulp fibers has to be changed; this change is accomplished by depositing a positively charged polymer as a binder. The cationic polyacrylamide (c-PAM) with a degree of substitution of 20% with a dosage of 1 mg/g of paper, or PIDMAC, with a dosage of 12 mg/g were used as binders. These dosages correspond to full coverage of the binder on the fibers. The amount of PIDMAC is larger because of its lower molecular weight, which causes a substantial amount of PIDMAC to penetrate the porous fiber wall. The high molecular weight c-PAM remains all on the external surface of the fibers. Prior to the CPE binder deposition, each filter paper sample is weighed dry and afterwards presoaked in water in a petri dish and weighed again; this is done to determine how much water or solution can be absorbed per gram of dry paper. This information allows one to adjust the CPE concentration such that, at equilibrium absorption, the appropriate amount has been taken up. The next step consists of drying the soaked filter paper in air overnight.

After drying, the triclosan loaded block copolymer micelles with the negatively charged corona are attached to the positively charged filter paper. Since the volume of solution which can be absorbed by an individual sample of filter paper is known, the paper is now exposed to the micelle solution and a known quantity of micelles becomes attached to the paper via the interaction of the PAA in the micellar corona and the CPE coated fibers of the paper. The final concentration of the loaded micelles on the filter paper is 1 mg of micelles/g of paper, which roughly corresponds to a monolayer of loaded micelles on the pulp fibers. In the final preparatory step, the paper is dried in air overnight.

It should be noted that for each paper sample, controls were also prepared. The first control is the untreated paper without any additives, the second control contains paper coated only with CPE but without loaded micelles, and the third control sample contains paper treated with loaded micelles without any CPE. Control tests for utilizing empty (unloaded) micelles were not performed since it had previously been found that empty micelles do not affect significantly the growth of bacteria.[16]

2.4. Efficiency of Antibacterial Filter Paper

2.4.1. Filter paper composed of c-PAM and triclosan-loaded micelles

To test the efficiency of the antibacterial filter paper, *E. coli* 11229 bacteria were first incubated overnight in a growth media (Mueller Hinton) at 37 °C while shaking. The concentrations of the bacteria solutions were determined by UV-vis for their absorbance and then diluted to an absorbance of approximately 0.1, which corresponds, according to the calibration curve given in the Supporting Information in the previous paper,[16] to roughly 25×10^6 bacteria/mL. The bacteria were then diluted with Milli-Q water to an absorbance of roughly 0.1 and continued to be incubated in the growth media while shaking at 37 °C for about 2 h to an absorbance of 0.3 to 0.5, to guarantee the freshness of the bacteria sample and to assure that the bacteria concentration is in the linear region (log growth) of the growth curve. The bacteria solution was then diluted with milli-Q water to reach an absorbance of 0.1.

In the next step, the modified filter paper, containing both CPE and triclosan loaded micelles, was placed into a funnel, and attached to a stand below which there is a beaker to collect the filtrate. A given volume of the solution of bacteria of absorbance of 0.1 was passed through the filter paper and was collected as a filtrate in the beaker below the funnel.

If the filter is efficient, the bacteria should be deactivated after the bacteria solution is passed through the filter paper. Therefore, the efficiency of the filter paper was tested in the following way: After passing through the filter paper, 1 mL of the filtrate bacteria solution was mixed with 9 mL of growth medium to achieve ideal growth conditions for the bacteria. The bacteria solution was then incubated for 3 h in a shaking incubator at 37 °C while the absorbance was monitored as a

function of time. If the absorbance of the filtered bacteria solution in the growth medium continues to increase, the bacteria are multiplying and the filter paper is considered inefficient. If the absorbance of the filtered bacteria solution in growth medium does not increase, the bacteria are not multiplying and the filter paper is considered successful and efficient in bacteria deactivation. The above mentioned procedure was repeated also for the control samples, containing only CPE binder, only loaded micelles and also for filter paper without any additives while testing various experimental conditions, one at the time. Triplicates of each filter paper sample were prepared.

The filter paper is considered to be efficient if the relative absorbance after 3 h of growth did not increase from the original value of 1.00 to over the value of 1.03, as typical variations in absorbance for non-growing bacteria are 3%.[17] All the UV-vis experiments were done at a wavelength of 600 nm.

The parameters of the experiment were the following: Whatman filter paper, 90 mm in diameter, grade 3, with a pore size of 6 μm was used. The concentrations of c-PAM and triclosan loaded micelles was 1 mg/g of paper for each, which roughly corresponds to monolayer coverage of micelles on the fibers. The concentration of the micelles in the solution was 1 g of polymer/L. The biocide loading was approximately 20 wt% of the weight of polymer; i.e. a total loading of 1 gram of micelles/L corresponds to 0.2 g of biocide/L. The absorbance of the bacterial solution was 0.1, which corresponds to roughly 25×10^6 bacteria/mL; the total volume of bacterial solution passing through the filter was 100 mL.

2.4.2. Filter paper composed of PIDMAC and triclosan-loaded micelles

A single colony of the *E.coli* 11229 strain was grown overnight in 85 mL of sterile MHB (Mueller Hinton Broth) solution placed in a 250 mL flask. 300 μL of this overnight culture was added to two (or more as needed) new 85 mL sterile MHB solutions in 250 mL flasks (with absorbance 0.5–0.6) and incubated in a 37 ºC water bath shaker (150 rpm) for 2 h. Following this procedure, the bacteria were in the linear phase of growth as shown in a growth curve experiment that was previously conducted, the results of which were shown in the Supporting Information in a previous paper.[16] The bacteria suspension was then diluted to absorbance ≈0.1 with sterile deionized water, at which stage it was ready for filtration. 50 mL of this *E. coli* suspension was passed through each filter paper sample using sterilized glass funnels and 50 mL conic sterile tubes or sterilized flasks. For this experiment the following filter papers were prepared: blank (untreated), coated with PIDMAC binder only, bactericidal filter paper coated with PIDMAC binder and triclosan loaded micelles and coated with PIDMAC and empty micelles. Triplicates of each filter paper sample were prepared.

In order to determine the concentration of bacteria that survive in the filtrate, 100 μL of each filtrate sample was plated on nutrient agar plates after appropriate dilution (absorbance 0.1). For very low bacteria concentrations no dilution was performed. Bacteria counts in an unfiltered bacteria suspension were done as a second control, which yielded values always similar to those of the original control. The nutrient agar plates were incubated overnight at 37 °C (≈18 h), and the colony number was counted (any counts under 30 colonies are considered TFTC, that is too few to count, a common procedure.[18] Results are presented as the average count of triplicate filtrates.

Bacteria growth was followed as well. Absorbance of the filtrates was measured at 600 nm. The growth ability of the bacteria in the filtrate was tested after normalization of all samples to the lower absorbance (by dilution with similar combination of MHB and milli-Q wanter in the filtrate) followed by 10 times dilution with fresh MHB in 15 mL conic sterile tubes. In addition to the filtrate sample, a sample of unfiltered bacteria was prepared as a control. The lids of tubes are released to allow air and all samples are incubated in a 37 °C water bath shaker. Growth of the *E. coli* was followed by absorbance every hour for the next 3 h. Results were presented as relative absorbance, after taking into consideration all dilutions done for normalization. Results were the average of triplicates.

3. Results and Discussion

3.1. Efficiency of Antibacterial Filter Paper

The efficiency of the filter paper modified with c-PAM and the triclosan loaded PS-b-PAA block copolymer micelles was evaluated. The modified filter paper was placed into a funnel and the *E. coli* solution was passed through the filter paper. Afterwards, the filtrate was tested for bacteria growth. This procedure was repeated also for three control samples; for plain filter paper without additives, for filter paper modified only by c-PAM and for filter paper modified only by triclosan loaded micelles.

The results of the experiment are shown in (**Figure 1**) as a function of relative absorbance of bacteria incubation time. It is obvious that the plain filter paper without any additives (control sample) does not hinder the growth of the bacteria, since the relative absorbance increases from an initial value 1 to about 7 within 3 h.

It is also clear from the figure that bacteria which passed through the filter paper modified with both c-PAM and triclosan

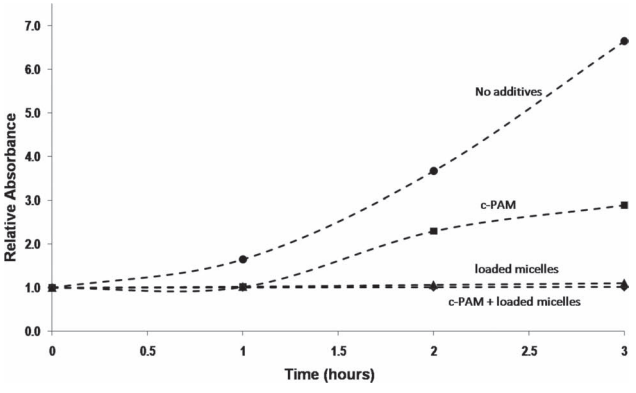

Figure 1. Efficiency of the variously treated antibacterial filter papers for *E. coli* solutions passing through a grade 3 Whatman filter paper with a pore size of 6 μm. The experimental conditions were the following: concentration of both c-PAM and triclosan loaded micelles was 1 mg/g of paper; 100 mL of bacteria solution of absorbance of 0.1; filtering time was between 30 and 60 min, depending on the treatment of the filter paper.

loaded micelles do not grow with time at all, since the relative absorbance does not increase above 1. Thus, the antibacterial filter paper treated with c-PAM and triclosan loaded micelles is efficient in deactivation of *E. coli* bacteria at a concentration of 25×10^6 bacteria/mL.

A result similar to that obtained for the filter paper modified by both c-PAM and triclosan loaded micelles is achieved by modifying the paper with only triclosan loaded micelles. In this case, the bacteria also do not grow with time, as is evident from the figure. The disadvantage of such treated filter paper is that the loaded micelles are not attached to the filter paper and, thus, are passing through the filter paper, along with the bacteria solution. It should be recalled that the charge on both the filter paper and the micelles is negative, thus preventing electrostatic attraction between them, in contrast to the situation with c-PAM coated fibers. Therefore, the bacteria become deactivated by triclosan released from the micelles during collisions in solution between the unattached loaded micelles and the bacteria which pass together through the filter paper. For practical applications, it is preferable to keep the micelles attached to the filter paper.

c-PAM by itself adsorbed on the pulp fibers acts as a week antibacterial agent; this can be deduced from the fact that the relative absorbance of a bacteria solution passing through such treated paper increases slowly with time; in the present test, the relative absorbance of a bacteria solution increased in 3 h from an initial value of 1 to approximately 2.5. By contrast, in a solution of c-PAM without micelles or fibers (previous publication[15]), the c-PAM adsorbs on the bacterial surface and deactivates the bacteria completely; the bacteria do not grow with time at all.[15] The c-PAM adsorbs on the bacteria because in solution the bacteria are negatively charged and c-PAM is positively charged. On the filter paper, the c-PAM is first adsorbed onto the negatively charged pulp fibers, and thus cannot adsorb as easily on the bacteria as they pass by. This is the reason why the c-PAM has a slightly different effect on bacteria in solution than when it is attached to the filter paper. Also, since the bacteria adhere to the pulp fibers, only a small fraction of the bacteria ends up in the filtrate. The filtering time in the present experiment was between 30 to 60 min, depending on the type of filter paper modification. The fastest filtration of 30 min was achieved by plain filter paper without additives. Under optimal conditions the drainage times for papers with and without micelles are similar. Ways to reduce the drainage time are addressed in a forthcoming publication.

It should be mentioned that the deactivation of the bacteria by the triclosan was demonstrated in the previous publication,[16] as supported by absorbance results and SEM images of the bacteria after deactivation. The mechanism of bacteria deactivation was also elucidated;[16] it was shown that the biocide is transferred to the bacteria by repeated micelle/bacteria collisions, and not via dissolved biocide. The mechanism of action of triclosan on the *E. coli* bacteria is known to be blocking of lipid synthesis in the bacteria.[19] In addition, since the c-PAM binder has also antibacterial properties, the bacteria are deactivated by the effect of this binders attached to the filter paper as they pass through the filter. Therefore, the bacteria deactivation is caused by a combination of both effects, i.e., the effect of the antibacterial c-PAM binder attached to the fibers and that of the biocide transfered from the loaded block copolymer micelles attached to the c-PAM.

The efficiency of the filter papers depends on the type of biocide incorporated into the micelles and on the type of the polyelectrolyte binder to which the micelles are attached on the paper fibers; efficiency can be enhanced by varying the choice of these parameters. In the present work one type of biocide and two types of CPEs were tested. Given the large number of bactericidal agents and very large parameter space in the application of CPEs (type of CPE, degree of coverage, M_w, degree of branching etc.) it is very likely that dramatic improvement in the already very high efficiency is achievable via a systematic optimization process

3.2. Synergy Between Biocide and Cationic Polyelectrolyte

Another combination of CPE and triclosan loaded micelles was tested. As an alternative CPE binder, PIDMAC was chosen to test the efficiency of the antibacterial filter paper. The bactericidal filter paper is made of Whatman filter paper grade 2, which has 110 mm diameter circle and 8 μm pore size. It was coated with 12 mg PIDMAC per 1 g of fibers and triclosan loaded micelles as described in the experimental section. Bacteria survival was tested after gravity filtration of bacteria suspension through this bactericidal filter paper and compared to filtration through blank filter paper, filter paper treated with PIDMAC and filter paper treated with PIDMAC and empty micelles. Results are shown in (**Figure 2**) where plate counts (a) and bacteria growth (b) after filtration through various filter papers are presented. Plate counts reveal around 7 orders of magnitude reduction in bacteria viability when filtered through the bactericidal filter paper. Therefore, TFTC does not indicate that no bacteria were found but specifies that the bacteria concentration is below 10^2 CFU/mL. In this experiment, counts were very low, thus, indicating that the bacteria concentration is in the range of 10^1 CFU/mL.

Filter paper treated with the cationic PIDMAC binder is very good in bacteria deactivation as it could reduce the number of live bacteria by 5 orders of magnitude. However, when empty micelles were added to filter paper treated with PIDMAC, the reduction was only 4 orders of magnitude. Probably, this is due to deposition of micelles on PIDMAC, thus neutralizing part of its bactericidal activity. The addition of triclosan has an additive bactericidal effect of more than 3 orders of magnitude. Bacteria survival treated with triclosan alone reduced bacteria viability by 1 order of magnitude. For PIDMAC-treated paper and comparing the addition of empty micelles to triclosan loaded micelles, one can see a substantial synergy when PIDMAC and triclosan are acting together.

The *E.coli* concentration of filtrate that was passed through blank filter paper is similar to that of the bacteria suspension before filtration, indicating that the cellulose fibers of the filter paper have no deactivation effect on the bacteria.

4. Conclusions

This paper describes the preparation of antibacterial filter papers designed mainly for small scale water purification. The

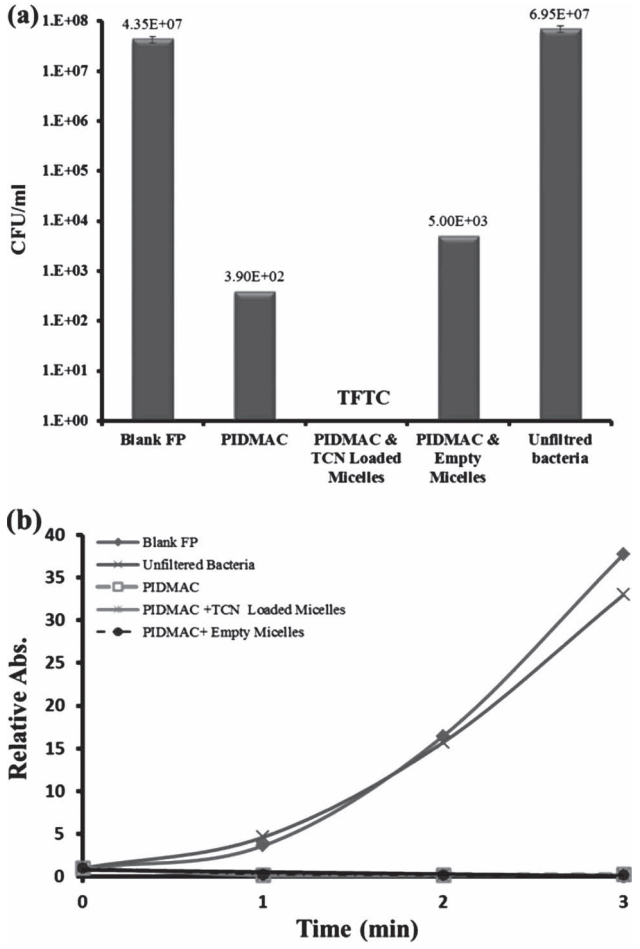

Figure 2. a) Efficiency of bactericidal filter paper for various filter papers, all made of Whatman filter paper grade 2 (110 mm diameter circle and 8 µm pore size). Plate counts of the filtrate are presented. TFTC is to few to count (under 30 colonies). b) Bacteria growth of *E.coli* of the same filtrates tested in (a). Growth was followed for 3 h after filtration.

preparation of the antibacterial filter papers involves a hydrophobic biocide, triclosan, which has a very low water solubility and which is incorporated into the hydrophobic core of amphiphilic block copolymer micelles, which, in turn, are attached to the fibers of the filter paper which are coated with a cationic electrolyte. Because a saturated solution of triclosan is not efficient in bacteria deactivation, a reservoir is required to increase its concentration and the micelles provide such a reservoir. In addition, a collisional transfer mechanism is operating to transport the biocide molecules to the bacteria without introducing micelles into the filtrate and hence into the body when drinking the filtrate.

We have shown that both the biocide (triclosan) and the CPE binder (PIDMAC) are the active components in bacteria deactivation. The combination of the effects of both biocides acting synergistically on the bacteria is responsible for the extremely high efficiency of 7 orders of magnitude reduction in live bacteria. The bacteria colony reduction achieved by our bactericidal filter paper is one order of magnitude higher than that recommended by US EPA standard, which requires the reduction of viable bacteria colony count by 6 orders of magnitude (log 6) for water purification devices.[19]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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