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Polythiophene Biosensor for Rapid Detection of Microbial Particles in Water

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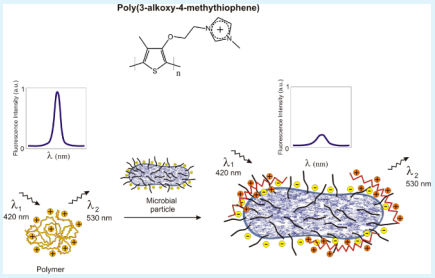
ABSTRACT: Most microbial particles have a negatively charged surface and in this work, we describe a water quality monitoring application of a cationic polythiophene derivative (AH-35) for the rapid assessment of microbial contamination of water. Using *E. coli* as a prototype microbial particle, we demonstrate that the AH-35 polymer can provide a qualitative assessment of water if exposed to more than 500 CFU/mL, thereby paving the way to a new family of biosensors potentially useful for monitoring drinking water distribution systems.



KEYWORDS: *cationic polythiophene, biosensor, drinking water, microbial particles*

1. INTRODUCTION

Water of good microbiological quality is essential for maintaining human health. Each day, diarrheal diseases attributable to unsafe water or inadequate hygiene/sanitation result in 4000−6000 deaths, mostly children.[1] Undoubtedly, the design and construction of water distribution networks constitutes a major technological development of the last century that greatly contributed to improve hygiene and reduce the occurrence of waterborne diseases, but there is evidence that (aging) distribution systems are increasingly contaminated by a wide range of microbes and/or associated with disease outbreaks.[2,3] Indeed, (tap) drinking water naturally contains viruses, bacteria, fungi, and parasites that have either (1) survived the sanitation process (filtration, chlorination, etc.), (2) been released from biofilms of the distribution system, household pipes or reservoirs, (3) proliferated in reason of extended retention or stagnation, or (4) entered the distribution system following a structural failure.[1,4−12]

The detection of fecal contamination indicators (FCI) is generally used to provide an evaluation of a relatively recent contamination of a water supply by fecal matter, a matrix highly susceptible to contain human pathogens. The risk assessment of the microbiological quality of water implies the detection (and quantitation) of FCI such as *Escherichia coli* and enterococci by culture-based procedures that (1) have a long turnaround time (18−72 h) especially if confirmation is done by culture, (2) are seldom inadequate to predict the presence of (non-culturable or fastidious) pathogens,[13−17] or (3) are prone to false-negative results since they have an imperfect ubiquity, ubiquity being defined by the ability to detect most if not all strains of a species.[18,19]

Methods based on membrane filtration of fixed volumes of water (usually 100 mL, but 250 mL volumes are tested in Europe) on cellulose membranes followed by culture of the bacteria trapped in those membranes constitute gold standard methods used to detect indicator or index microorganisms but, in many instances, most probable number (MPN) methods using defined substrate methodology in liquid medium (e.g., Colilert, Enterolert) are simpler and more economical.[14]

The heterotrophic plate count (HPC) is a general microbiological procedure used to assess the microbial integrity of a water distribution network and indirectly indicate the effectiveness of water treatment processes (pathogen removal).[20] HPC detection methods recover autochthonous bacteria after an incubation of at least 2−3 days at 22 or 35−37 °C.[21] HPC may recover bacterial pathogens such as *Pseudomonas*, *Acinetobacter*, or *Klebsiella*, but not several fastidious or "viable but non culturable" (VBNC) microorganisms that might constitute health threats for immunocompromised individuals.[4−6,22,23] The modeling of HPC in a water distribution system has demonstrated that the best predictors of HPC in the system are (1) free residual chlorine (inverse relation), (2) water temperature, and (3) water ultraviolet light absorbance.[25] Although regulations stipulate that drinking water must be free of fecal contamination indicators such as *E. coli* i.e. 0 colony forming unit (CFU) per 100 mL, maximum accepted HPC contaminant levels range from 100 CFU/mL in Europe to 2000 CFU/mL in Japan, whereas in Canada and the United States, drinking water is considered unsafe if the HPC exceeds 500 CFU/mL.[3,10,22,24,25]

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Drinking water is an environment into which microorganisms will not easily thrive and the human health risk posed by non culturable or fastidious microorganisms and cells that have entered in the VBNC state[26−31] would warrant the development and implementation of adaptable, sensitive, specific, cost-, and time-effective methods for the detection of pathogens in water sources, especially those for which fecal contamination indicators are inadequate in predicting their presence.[32−34] To address this need, only PCR-based methods have the potential for the specific detection of pathogens.[17]

However, in the context of the work presented therein, the measurement of ATP, bacterial endotoxin (LPS), or fluorescent dyes, and cytometry, cell sorting, or FT-IR methods are also used or under evaluation to assess general microbial contamination or the metabolic status of microbial contaminants.[23,34−39] In this respect, ATP can provide a general measure of microbial cell viability, whereas the rapid detection of endotoxin can be used to assess the presence of gram-negative bacteria and fungi. Compared to the application of cationic polythiophene biosensors described in this work, these methods have a limit of detection generally estimated >100 CFU/mL, but are severely limited in terms of specificity.

Water-soluble cationic polythiophene biosensors (CPB) are macromolecules with highly desirable properties for the sensitive optical detection of negatively charged macromolecules such as nucleic acids and proteins. The electrostatic binding of CPB to negatively charged macromolecules induces conformational and solubility changes leading to an optically measurable signal. In fact, uncomplexed CPB polymers, presumably in a random coil conformation in the native state, tend to rearrange into a more organized state upon binding to the negatively charged phosphate backbone of DNA for example, thereby inducing a chromic transition measurable by methods such as colorimetry or more sensitive fluorescence spectrophotometry.[40−50]

The outer ultrastructures of microorganisms such as bacteria and environmentally resistant forms of waterborne parasites are generally negatively charged at neutral pH[51−53] and there are a few examples in the literature describing the interactions of cationic polymers with whole cells.[54−56]

In this work, we describe the use of a cationic water-soluble polythiophene for the rapid and direct assessment of the microbiological quality of water based on the HPC criteria applied in the United States and Canada. However, since this method could be used to detect microbial particles that are either in the VBNC state or not recoverable by culture media used for detecting HPC bacteria, we have based our approach on an operational criteria of 500 microbial particles (MP) per mL. The method we have developed is based on the optical fluorescence measurement obtained following polymer conformation transitions and/or interactions upon binding to the outer ultrastructure of bacterial cells. In contrast to current water quality assessment methods, it does not require cultivation nor molecular amplification of microbial nucleic acids.

2. EXPERIMENTAL SECTION

2.1. Synthesis, Purification, And Characterization of AH-35 CPB. The water-soluble cationic polythiophene polymer **AH-35** (Figure 1) was synthesized according to Ho et al.[40] As reported earlier, its chromic properties principally result from conformational changes of the flexible conjugated backbone.[44]

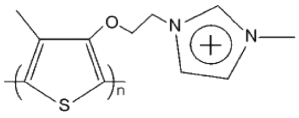

Figure 1. Structure of cationic polythiophene polymer (AH-35).

2.2. Bacteria and Culture Conditions. *E. coli* ATCC 11775 were cultivated in brain heart infusion (BHI) broth medium (Difco) at 37 °C. *E. coli* cells were grown to late log phase in BHI broth ($OD_{600\ nm}$ of 1.83); the optical density of the bacterial culture was determined with a cell density meter (Model 40, Thermo Fisher Scientific Company). The bacterial cell concentration was determined using a Petroff-Hausser counting chamber and confirmed by spreading onto blood agar solid medium. Aliquots of the bacterial culture were diluted in reverse-osmosis purified (nanopure) water to obtain cell suspensions calibrated at 1×10^7 CFU/mL for the biosensing assays. The suspensions were stored at 4 °C until use.

2.3. Biosensing Assay. The fluorescence measurements were taken on a Varian fluorescence spectrophotometer (Agilent Technologies). The excitation wavelength was set at 420 nm and the emission range, between 500 and 650 nm. Both emission and excitation slits were 10 nm wide. The fluorescence emission spectrum of 3 mL of nanopure water was taken as the method blank. Then, 1.38×10^{-7} M (4.4 μL) of polymer was added and the optical cell was maintained at 20 , 22, 37, or 45 °C for 3 min before the spectra was recorded. Then, a specific volume of the concentrated suspension of bacteria was added to get the desired concentration (1000, 500, or 100 CFU/mL.) Then, another spectrum was recorded after a 3-min incubation at the desired temperature. The experiments were repeated five times the same day with the same suspension of microbial particles.

3. RESULTS AND DISCUSSION

As briefly said in the introduction, the cationic polythiophene (AH-35) is known to show a diminution of its initial fluorescence when in the presence of polyanionic macromolecules (like DNA or RNA) or negatively charged particles. It also presents a wavelength shift in fluorescence and in colorimetry when its conformation changes from a nonplanar form to a planar one. In contrast, Figure 2 suggests that the interactions between the AH-35 polythiophene biosensor and intact microbial particles do not induce a fluorescence wavelength shift but rather a decrease in fluorescence intensity, indicating that a biophysical phenomenon such as physical quenching enable biosensing.

3.1. Microbial Cell Biosensing. Figure 3 shows that in the presence of bacterial cell loads in excess of 500 MP/mL and at a temperature of 20 °C, a diminution of the fluorescence of the AH-35 CPB signal is observed, suggesting that the attractive ionic interactions between the cationic sensing element and the polyanionic scaffold provided by the bacterial cell surface may quench the fluorescence emitted by the CPB. A similar effect on fluorescence intensity was also reported by Panda et al.[55] upon binding of gold nanoparticle−polythiophene composites to the cell surface of *Pediococcus acidilacti* and *E. coli*, but the decrease in fluorescence intensity did not appear as significant as what we observed.

Not shown in Figure 3 are experiments done in the presence of 1×10^4 MP/mL. In these experiments, the fluorescence of the polymer was extremely low, suggesting that the AH-35 polymer is totally quenched or adsorbed by the microbial particles and that an important microbial contamination in water could be easily detectable using this method.

Indeed, when the polymer is in solution, it presents a random coil conformation yielding an intense fluorescence peak at 530

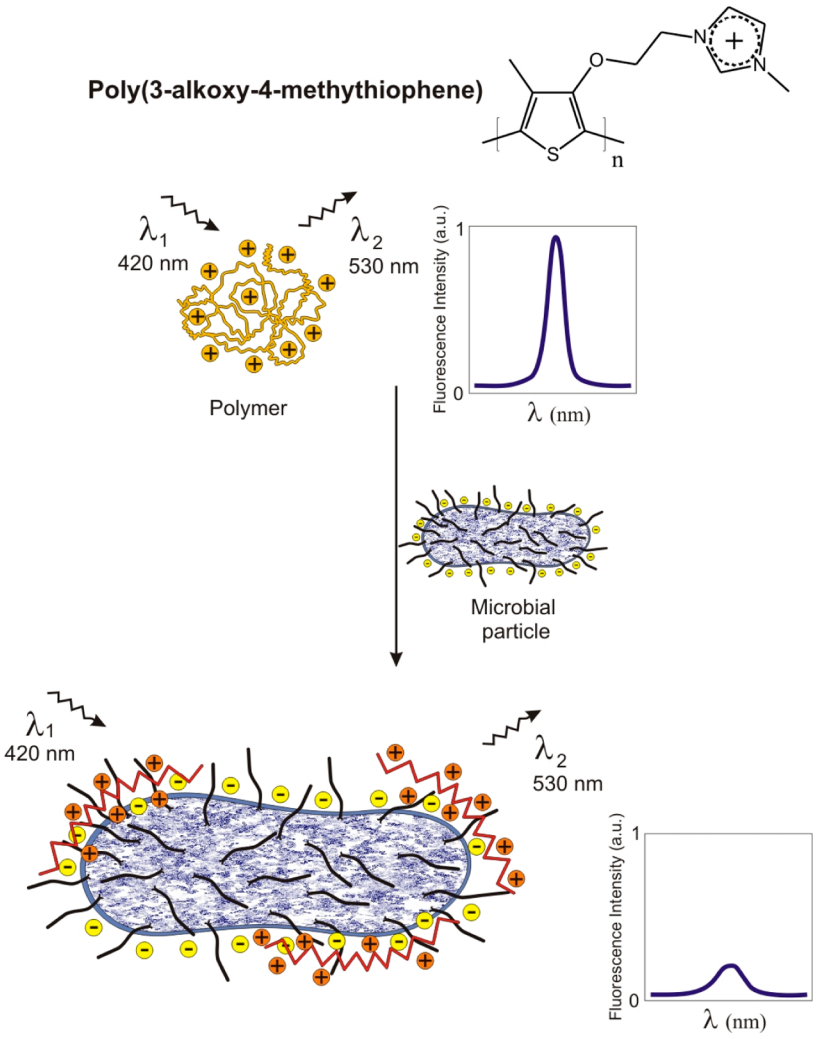

Figure 2. Schematic description of the method used. When the CBP is alone, its fluorescence is optimal. After adding a microbial particle, a diminution of fluorescence without any wavelength shift is notable.

nm.[40] For the foreseen application of the AH-35 CPB in the assessment of the general microbiological quality of water, these results are very significant because they demonstrate a statistically different signal at and over the HPC criteria recommended in Canada and the United States (500 CFU/mL).

However, the development of a commercial biosensor system might be hampered by the fact that a concept instrument shall uncover a decrease in the fluorescence intensity. This decrease might be explained that, upon binding to the negative charges of the bacterial cell wall, mainly those of the highly abundant membrane phospholipids,[57] a significant portion of CPB macromolecules are sequestered at the surface of the cell wall and the generation of the fluorescence signal (excitation and/or emission) might be affected or diminished by other outer membrane components such as proteins and lipopolysaccharides protruding outward, thereby leading to a lesser intensity of the fluorescence instead of a chromic shift generally observed upon rearrangement on a polyanionic electrolytes or macromolecules.

3.2. Influence of Temperature on the Biosensing System. Twenty-two and 37 °C are incubation temperatures generally used for growing HPC.[21] The influence of those incubation temperatures, as well as 20 °C (generally called room temperature), was evaluated in the presence of increasing bacterial loads, from 0 to 1000 CFU/mL. Figure 3 shows that increasing the incubation temperature of the biosensing system decreases the variation of the fluorescence signal data points and allows for an enhanced discrimination of the spectra but this is not significant.

According to Ho et al.,[40] the chromic properties of the polymer are optimal when used at 55 °C, but *E. coli* are not viable above 45 °C. The best compromise was therefore to perform fluorescence measurements at a temperature that could be tolerated by bacteria (before temperature-induced lysis). That temperature, even if it is not optimal, should reduce fluorescence signal fluctuations of polymer. In these conditions, the discrimination between the polymer used alone or in the presence of bacteria appears more significant, suggesting that the biosensing system could effectively indicate if water is contaminated with more than 500 MP/mL (Figure 3). It is known that the fluorescence of AH-35 might be influenced by negative chemical ions such as Br^- or I^-,[44] but there are fewer data regarding the influence of chemical or organic polyanions (biofilm metabolites) as well as residual chlorine and chloramines at concentrations found in drinking (tap) water. The influence of these potential contaminants on the biosensing system described in this work shall be investigated.

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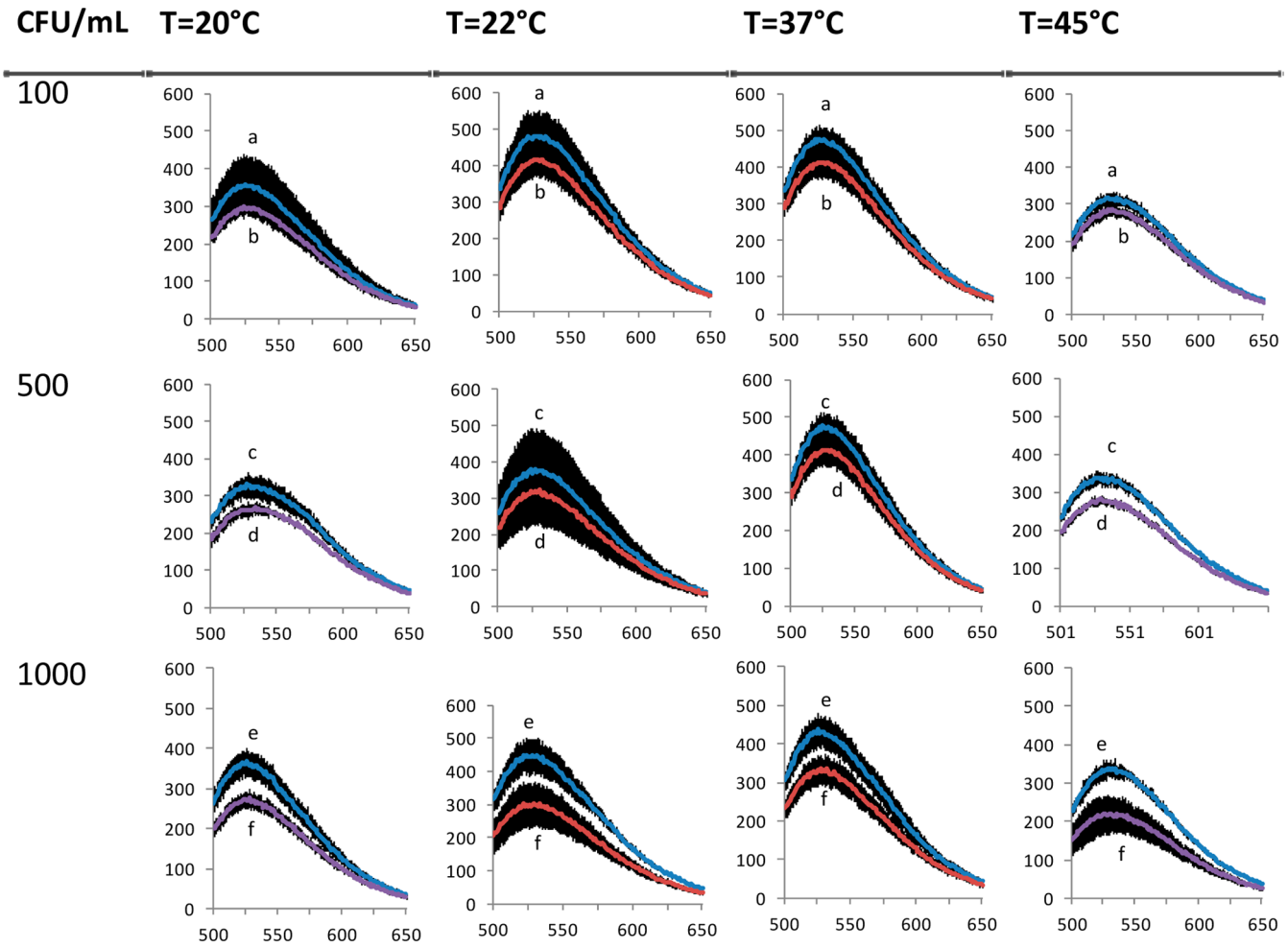

Figure 3. Fluorescence intensity (arbitrary units) of the AH-35 polymer in function of the wavelength, alone (1.38×10^{-7} M in nanopure water); graphs a, c, and e) for 100 (graphs b), 500 (graphs d), and 1000 (graphs f) CFU/mL of *E. coli*.

In contrast to the chromic transitions generally observed when CPB interact with polyanionic species, the higher temperature of the biosensing system does not affect the fluorescence peak wavelength, but improves signal stability.

This rapid method for detecting microbial particles in water could also find applications in dental clinics or in other medical centers, for testing dental unit water systems (DUWS) or water used for renal dialysis. The required equipment would consist of a portable fluorescence spectrophotometer set on fixed excitation and emission wavelengths and a predetermined temperature, and of 1 mL UV-compatible cuvettes coated with a specific quantity of the cationic polymer. After a short incubation time, the sensing system would provide a positive signal if the MP concentration exceeds 500 MP/mL and, hence, if the water is safe to use (drink) or not.

4. CONCLUSIONS AND PERSPECTIVES

In contrast to standard methods developed for the determination of fecal contamination indicators, the heterotrophic bacterial count cannot be used for assessing the risk of waterborne diseases but it can provide highly useful information regarding the efficiency of drinking water disinfection processes, as well as for assessing the general microbiological quality of water distributed in municipal, industrial, or other specialized (medical) networks. However, because HPC determination is a culture-based method, it displays limitations associated with the in vitro culturability of microbes that can be recovered, as well as for its specificity with regards to the detection of other classes of microbes such as viruses and parasites.

In this work, we describe an application of cationic polythiophene biosensors for the culture-independent, direct, rapid, and yet extremely simple detection of microbial particles in water. For the time being and although the work described here was only accomplished with the gram-negative bacterium *E. coli*, the prototype microbial sensing system provides a statistically significant fluorescent signal when the microbial particle titer exceeds 500 MP/mL, the operational criteria observed in Canada and the United States. Integrated in a (automated) system, we believe that CPB-based determination of microbial particles in drinking water could be tested in parallel with that of residual chlorine and serve as a monitoring tool for the general microbiological quality of water distribution networks or as part of coordinated strategies for the rapid detection of microbial intrusion.[58,59]

Future developments of the biosensing system include (1) testing the CPB in the presence of other (or combinations of) microbial particles such as bacteria, lower eukaryotes (parasites, amoebas, etc.) and perhaps, viruses frequently recovered from drinking water, (2) testing the CPB in tap and bottled drinking water sources to investigate the influence of contaminants other than microbes, and (3) designing a "positive" detection method

(and reagent) to simplify the "calling" of a water sample exceeding 500 MP/mL.

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Notes

The authors declare no competing financial interest.

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