Research Article

BIOMEDICAL Journal of Scientific & Technical Research

ISSN: 2574 -1241

DOI: 10.26717/BJSTR.2020.32.005219

New Antimicrobial Devices to Prevent Waterborne Diseases

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ARTICLE INFO

Received: November 23, 2020

Published: November 30, 2020

Citation: G Palmieri, A Cammarano, B Agrillo, YT Proroga, M Gogliettino, et al., New Antimicrobial Devices to Prevent Waterborne Diseases. Biomed J Sci & Tech Res 32(2)-2020. BJSTR. MS.ID.005219.

Abstract

The consumption of bottled water has been increasing consistently over the last decade, even in countries where tap water quality is considered excellent. It has been known that there is an extremely high level of bacteria content and rapid microbial growth in reusable drinking water bottles, which have become more used by children and adults daily everywhere. Therefore, it is necessary to have some control strategies to reduce the bacteria level and minimize the associated health risk of the disease spreading. In this context, the development of functionalized polymer surfaces using bioactive compounds has grown rapidly over the past decade within many industries including biomedical, food packaging and beverage sectors. In this study a simple yet effective strategy to immobilize the de novo designed Antimicrobial Peptide (AMP) RiLK1 on PLA-surface's straw was developed. The RiLK1-immobilized PLA tubular surfaces displayed potent bactericidal properties against three of the most relevant waterborne pathogens such as *Escherichia coli, Salmonella Typhimurium*, and *Listeria monocytogenes*, demonstrating that RiLK1 retained its antimicrobial functionality also upon immobilization. To the best of our knowledge, this is the first proof-of-concept study that demonstrates the antibacterial effectiveness of a real tubular device functionalized with an AMP that could reduce or eliminate potential risks associated with water-related contamination.

Introduction

The rapid increase of highly resistant pathogens represents a global challenge in the food and beverage industry, as well as in the medical sector. In this context, one of the most important problems is correlated to the risk of pathogen exposure in the water, which represents a vital resource for the health and the survival of humanity. Generally, the greatest microbial risk to public health is associated with the consumption of contaminated drinking water. Indeed, the World Health Organization (WHO) reported that 884 million global population lack access to clean water and that 2.2 million deaths, mainly children, are attributable to diarrhea, which is transmitted through contaminated water, sanitation, or inadequate hygiene [1,2]. To overcome these problems, the demand and the consumption of bottled water have steadily increased during recent years, making it the fastest-growing category of soft drinks worldwide. Bottled water is recommended in hospital patients with immune deficiency to prevent the onset of possible diseases. Although the microbiological analyses often detect the presence of several microorganisms in bottled water, such as bacteria, most of them do not cause diseases at low concentrations. Elevated levels of microorganisms could pose a risk to vulnerable populations such as pregnant women, new-borns, immunocompromised patients, and the elderly [3].

From a strictly objective perspective, bottled water is not necessarily "better" or "worse" than tap water – it depends on the specificity of particular cases. Indeed, comparative studies between bottled and tap water demonstrated that bottled water is often no healthier than tap water [4-7]. Moreover, the usage of plastic leads to an increasing amount of waste and soil pollution. Therefore, it is spreading the use of water bottles made of reusable plastic materials, such as copolyester, polycarbonate, polyethylene, polypropylene, steel, and aluminium, to overcome this problem and contribute to saving the environment. In this context, although the reusable drinking water bottle is normally used daily for many months or even years by the consumers, little information relating to the microbial growth in this container and its associated health risks is available [8]. Nowadays, water bottles are acquiring an ever-growing interest in the market, which involve the use of drinking straws, thin hollow cylinders of paper, bamboo, stainless steel, or plastic (such as polypropylene, polystyrene, and polylactic acid) that allow the consumer to drink a beverage more conveniently. The straws when in direct contact with hand, mouth, or other sources of contamination, create an environment even more favorable to the growth of microorganisms, posing a health risk to people. Therefore, the research is more interested in exploring and developing innovative strategies for improving the preservation of drinking water, maintaining both the safety and stability of beverage by reducing or eliminating spoilage pathogens.

In this context, one of the most interesting strategies, which are effective in reducing the onset of pathogenic bacteria, involves the use of Antimicrobial Peptides (AMPs). They are indispensable components of the innate immune system in all living organisms and represent the first-line defence against a wide range of invading pathogens such as viruses, bacteria, and fungi [9]. Moreover, they exhibit a low probability to develop antimicrobial resistance likely thanks to their membrane-associated activity. Indeed, AMPs are part of a chemically and structurally heterogeneous family of very close-related peptides, sharing substantial primary sequence and physico-chemical similarities, such as small size, propensity for helical structuring, amphipathicity, and highly cationic character. Such physical characteristics allow AMPs to efficiently interact with the negatively charged microbial membranes and penetrate into cells via a detergent-like disaggregation, thus leading to membrane lysis and cell death [10]. In recent years, AMPs are considered excellent coating candidates for developing a myriad of antimicrobial biomaterials because they are far less susceptible to enzymatic degradation and to the development of pathogen resistance compared to conventional antibiotics, they exhibit rapid and broad-spectrum killing profiles, long-term stability and they are effective at low concentrations avoiding toxicity.

However, it is critical that peptide tethering to solid supports does not compromise its orientation, flexibility and thus its antimicrobial performance. Several AMPs have been coated successfully on a variety of substrates, which include contact lenses, glass, titanium oxide, silicone, resin beads, and silicon surfaces [11,12]. Nevertheless, the coating of AMPs is still challenged by suboptimal coating strategies leading to inadequate surface concentrations, tedious chemical reactions, and loss of antimicrobial activities with non-specific immobilization chemistry culminating in changed orientations of the peptide molecules and/or associated host cell toxicities. To address these problems, there is an urgent need for a combination of an efficient AMP candidate and an effective surface tethering strategy that would impart the desired antimicrobial characteristics on the targeted biomaterial. Recently, a novel decapeptide named RiLK1, was in silico designed and characterized, starting from the sequence of the previously studied 12-residue cathelicidin-related AMP named 1018-K6 [13-15]. RiLK1 was highly effective against both fungal pathogens and Gram-positive and Gram-negative bacteria, including multidrug-resistant clinical isolates of Salmonella, with no evidence of cytotoxicity on human cell lines.

In addition, RiLK1 was covalently conjugated to a model polymeric surface, revealing to efficiently prevent the growth of microbial spoilage also upon immobilization on solid support [13]. In this study, a functionalization strategy aiming at covalently grafting the antibacterial agent RiLK1 on PLA surface's straw was reported. In order to achieve this, plasma activation was used for improving the surface adhesion properties of PLA and promoting the surface binding of the peptide by the creation of more reactive groups on the inert polymer, thus making it suitable for the subsequent chemical functionalization. Therefore, the antimicrobial performances of the projected AMP-coated straws were determined against three of the most relevant waterborne pathogens such as *Escherichia coli*, *Salmonella Typhimurium*, and *Listeria monocytogenes*.

Material and Methods

In Silico Design and Synthesis of RiLK1

The peptide RiLK1 (NH2-RLKWVRIWRR-CoNH2, Purity: ≥95%) was purchased from GenScript Biotech (Leiden, Netherlands). RiLK1 was stored as a lyophilized powder at -20 °C. Prior to experimentation, fresh solutions in 100% DMSO were prepared, briefly vortexed, sonicated and these samples were used as stock solutions in all experiments. For all experiments the final peptide concentration is between 10-50 μM.

Atmospheric Plasma Treatments

Openair-Plasma® Technology was used as plasma treatment. The samples of PLA drinking straws were placed on the plate and the distance between the nozzle was set to 3cm. In all treatments, air was used as the processing gas with the power of 45 watts and a speed of 30 mm/min.

Characterization Techniques

Plasmatreat test Ink (Plasmatreat) was used to test the hydrophilicity of straws within a range of 48 and 72 mN/m.

Immobilization Yield Analysis of RiLK1 on Polymers

Immobilization yield investigation of RiLK1 on plasma-activated polymeric materials was performed by using a reverse-phase high-performance liquid chromatography (RP-HPLC) system. The polymer samples were immersed for 24h at 25 °C in 10 mM Na-phosphate buffer pH 7.5, containing RiLK1 at 50 µM concentration. Once coupling reaction was completed, the supernatant solutions were recovered and chromatographically analysed to indirectly estimate the amount of the peptide attached on the polymeric surfaces. For these analyses, 200 µL of the samples were injected over a µBondapak C18 reverse-phase column connected to a HPLC system, using a linear gradient of 0.1% TFA in acetonitrile from 5 to 95%. A reference solution was prepared with the initial peptide concentration used for the functionalization procedure under the same reaction conditions and run in parallel. Therefore, by knowing the added peptide (reference solution), the amount of peptide not bound to the polymers (expressed as a percentage) was determined by comparing the peak area. A calibration curve of the C18 column using different RiLK1 concentrations was built. All measurements were performed in triplicate on three different preparations.

Release Test

To determine the stability of the RiLK1-immobilized polymers, a release assay was performed by RP-HPLC system using a µBondapak C18 column applying a linear gradient of 5–95% acetonitrile in 0.1% TFA, at a flow rate of 1 mL/min. After coupling reaction, the peptide-immobilized polymer samples were cleaned by washing thoroughly in deionized water to remove all the unbound peptide and then immersed in pure water for 24 h at 4 °C or 25 °C. The recovered solutions were loaded onto the C18 column. The solutions in contact with the functionalized polymers at time t = 0 were used as control samples and run in parallel. All measurements were performed in triplicate on three different preparations.

Microbiological Assays

The microbiological assays were performed using PLA-drinking tubes functionalized with RiLK1 following the immobilization procedure described above. *Escherichia coli*, *Salmonella Typhimurium*, and *Listeria monocytogenes* LM2 (serotype 4b) [14] strains were all isolated from food products. Bacterial cells were cultured in BPW growth medium (Thermo Fisher, Milan, Italy) at 37 °C until collection and then diluted in water to a final concentration of 15–150 CFU/mL (CFU, colony forming units). Thereafter, a piece of immobilized straw (2.0 cm height) was immersed in 3 mL of drinking water contaminated with each bacterial suspension. The microbiological analyses were performed on contaminated water after 6 h and 8 h in contact with the RiLK1-straws. Samples containing cell suspensions and not-functionalized straws were used as control. Therefore, 50 µl cultures were plated on selective agar plates (*L. monocytogenes*, Agar Listeria acc. to Ottaviani & Agosti (ALOA) —Biolife Italiana; *S. Typhimurium*, Salmonella Chromogenic agar—Oxoid UK; *E. coli*, TBX agar—Biolife Italiana) and incubated for 24-48 h at 37 °C for *L. monocytogenes* and *S. Typhimurium*, while *E. coli* was incubated overnight at 44 °C. The plate counting method was used to estimate the bactericidal activity of the samples. Specifically, the number of colonies grown on agar plates in the presence of functionalized or not PLA straws were counted and compared. The analyses were performed in triplicate on three different experiments.

Results and Discussion

Our work has been focused on finding the best strategy to prevent bacterial contamination on polymeric tubular conduits by using Antimicrobial Peptides (AMPs), which are characterized by a broad antimicrobial spectrum activity and a low propensity for developing bacterial resistance, thus representing a suitable alternative to conventional antibiotics [16,17]. For this reason, the AMPs can be adequate to kill or inhibit the growth of microorganisms in contact with the external wall surface of polymeric tubular conduits like drinking straw. Indeed, the use of tubular conduits needs for their specific function handling or contact of their external surfaces, resulting in deposition of microorganisms, which may be subsequently transferred into the user's mouth and thus infecting the customer. To prevent microbial contamination of water contained in bottles with straw, our approach consisted in the functionalization of the straw's surface with the previously characterized antimicrobial peptide RiLK1, to obtain peptide-active tubular conduits. To this aim, commercial Polylactic Acid (PLA) drinking straws were chosen to allow the covalent attachment with the antimicrobial peptide. PLA is currently the most interesting biodegradable and compostable thermoplastic polyester derived from renewable resources such as corn or tapioca roots in contrast to the other thermoplastic materials which are petroleum-based [18,19].

Its biocompatibility and the consumers' desire to use a less impactful material have triggered its rapid entrance to the plastic market such as in the beverage industry for a variety of applications. However, disadvantages as hydrophobicity and the intrinsically low wettability limit its use as material in many cases such as for the functionalization with bioactive molecules. Therefore, in order to improve its surface features in terms of surface energy and adhesion properties without compromising the original bulk characteristics of the polymer, many approaches have been employed including physical and chemical treatments [20,21]. In recent years, one of the most promising strategies for polymer surface modification is the plasma treatment that enables the formation of chemically

reactive functional groups (-COOH∗, -OH∗) onto the surface layer of polymers, allowing the subsequence covalent derivatization with those present in the biomolecules [22]. In this paper, wall straw's activation was performed by atmospheric plasma treatment, which gave the best results in terms of yield of the immobilized peptide. Specifically, plasma activation was performed using the air as operating gas, which was jetted on the PLA tubular external surface through a circular nozzle with a power of 45 watts, a speed of 30 mm/min, and at a nozzle-substrate distance of 3 cm (Figure 1A). To be sure that the whole circumference of the straw was activated, the same procedure was repeated both after rotating the sample by 90 °C and on the flat straws.

The effect of the plasma treatment on the wettability of the PLA straws was assessed by using the test ink, a simple and quick method of estimating the surface tension of plastic materials. As shown in Figure 1B, plasma treatment produced an increase in the wettability of the polymer due to the creation of polar groups (C=O, C-OH, and R-COO−) on the surface in contrast to the untreated samples, which evidenced poor wetting. The measurement of the surface energy of our material was performed immediately after the plasma activation by using two test inks of known surface tension (72 mN/m and 48 mN/m), which were applied with a small brush. For definition, the surface tension of a material is equal to the value of the used test ink that remains intact for approximatively 2 seconds. If the edges of the liquid contract in less than 2 seconds after application, the surface tension of the material is lower than that of the ink, and the test must be repeated using an ink with a lower surface tension.

In our experiments, the brush stroke edges were stable for two seconds already with the test ink of 72 mN/m, which therefore corresponded to the surface energy of pre-activated PLA straws, confirming the strong increase of the surface hydrophilicity and thus of the wettability of polymer. These results were the opposite of those obtained before the plasma treatment, where the polylactic acid plastic had a lower surface tension than both inks, thus indicating its high hydrophobicity. The same experiments were also performed on the flat PLA surfaces to demonstrate that the geometrical structure of the straw did not affect the efficacy of the plasma treatment. As shown in Figure 1C, no discrepancy in the activation response was noted between the two types of structure, thus demonstrating that the tubular surface is well suited for the plasma treatment. Next, the covalent attachment of RiLK1 onto pre-activated PLA straws was performed by a one-phase immobilization process involving the simple immersion of the polymeric surfaces into the peptide aqueous solution at 50 µM concentration for 24 h, thanks to the creation of strong interactions between the plasma coatings and the peptide.

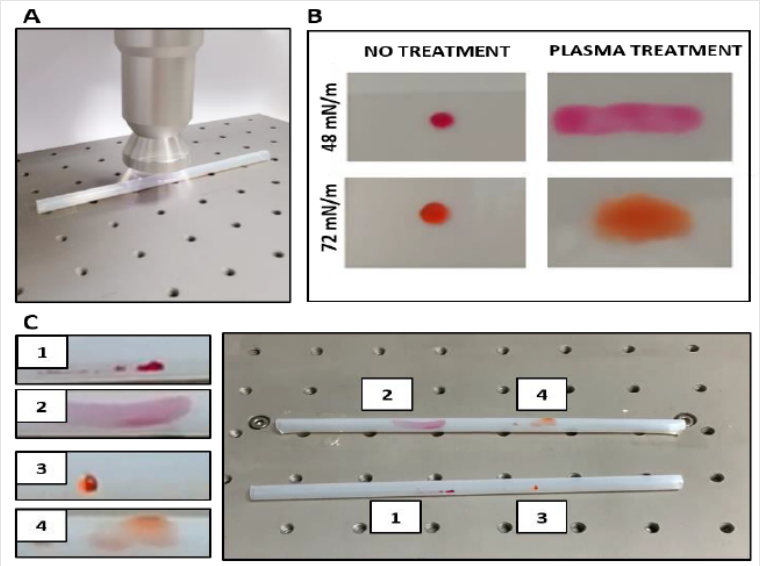

Figure 1:

a) Atmospheric Plasma Treatment on a commercial drinking straw made of PLA.

b) Comparison of 48 mN/m and 72 mN/m test inks on flat PLA, both on untreated sample and plasma treated sample (30 mm/min).

c) (Left) Zoom of the picture of straws treated with test inks to evaluate the surface tension. 1. violet ink (48 mN/m) on untreated straw; 2. violet ink (48 mN/m) on plasma treated straw. 3. orange ink (72 mN/m) on untreated straw. 4. orange ink (72 mN/m) on plasma treated straw. (Right) Comparison of 48 mN/m and 72 mN/m test inks on plasma treated and untreated drinking straws.

Indeed, the direct conjugation of AMP to the surface is favored by the formation of amide bonds between the chemically reactive groups (-COOH∗) generated on PLA by plasma treatment and the amine groups of the peptide. Therefore, the immobilization efficiency of RiLK1 on the polymeric device was quantitatively estimated by RP-HPLC analysis. In this experiment, once the conjugation reaction was completed, the supernatant solutions were recovered and analysed by RP-HPLC. In-depth, the amount of the peptide attached to the PLA was indirectly calculated by comparing the peak area of the unbound peptide, recovered after the coupling reaction, with the initial peptide concentration. The data obtained from these analyses demonstrated that the immobilization efficiency was 17%, corresponding to a surface coverage of 2.25 nmol/cm2 of PLA. In addition, the release of peptide from the polymeric tubes was evaluated in order to verify the stability of our system under actual conditions of use. Interestingly, no peptide-release occurred from the functionalized polymeric support during 24 h incubation, confirming the stable attachment of RiLK1 on the device via the covalent attachment, which not allows the peptide to withdraw from the surface, thus demonstrating a high stability of the tubular device (data not shown).

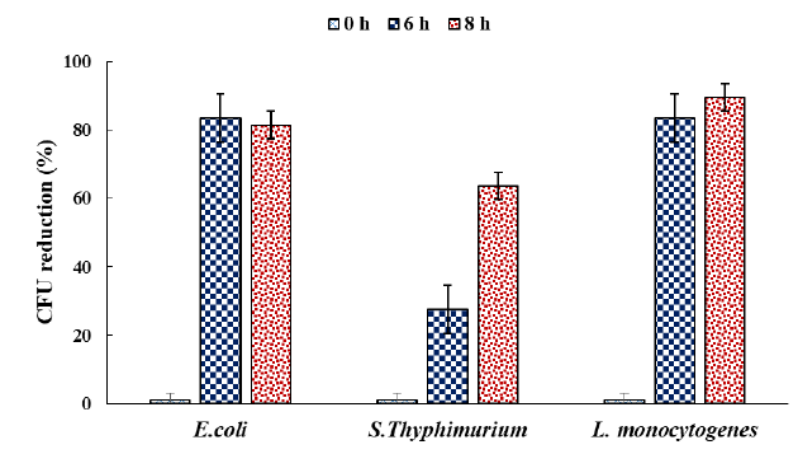

Figure 2: Effects of RiLK1-PLA on the microbiology quality of water. Antibacterial activity of RiLK1 tethered to PLA-straw against *E. coli, S. Typhimurium*, and *L. monocytogenes* at different incubation times.

CFU counting was performed to quantify viable bacteria. Data are presented as means ± SD of three different experiments performed in triplicate.

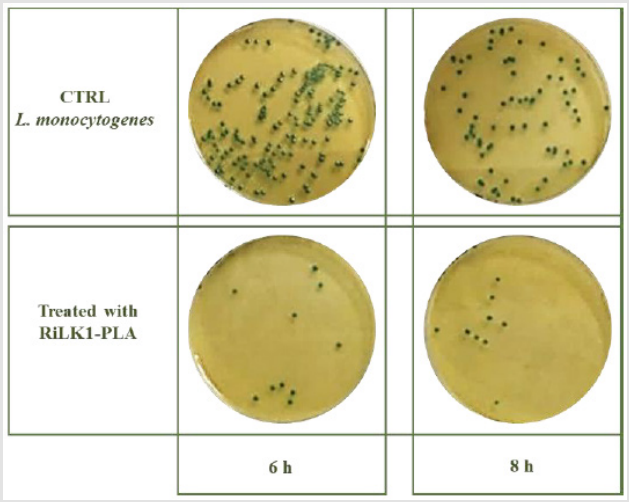

Figure 3: Representative bacterial plates of water samples contaminated with L. monocytogenes and incubated in the presence of not-functionalized PLA (CTRL) or RiLK1-PLA for 6h and 8h. The photographs are representative of three independent experiments performed in triplicate.

In order to assess the antimicrobial activity of the designed RiLK1-PLA tubes in contaminated drinking water, the microbiological quality of water was assessed over time by developing an experimental system that reproduced the real condition of usage of straws. Specifically, pieces of the functionalized surfaces were immersed in drinking water contaminated with three of the most common waterborne pathogens such as *Escherichia coli*, a multi-resistant strain of *Salmonella Typhimurium*, and

Listeria monocytogenes, using the non-functionalized straws as control. Briefly, the effect of treatment on bacteria was determined by counting the Colony Forming Units (CFU) on agar plates, which allowed an accurate determination of viable cells and the CFU reduction compared to the control was calculated in percent. As shown in Figure 2, RiLK1-devices displayed noticeable bactericidal properties against Escherichia, Salmonella, and Listeria strains after 6 h and 8 h of incubation, with a CFU reduction ranging from 30% to 80% and 60% to 90%, respectively. Representative bacterial plates for Listeria were reported in Figure 3. Altogether, these results emphasized the effectiveness of the immobilization procedure used in terms of both stability and antibacterial efficiency of the tubular device functionalized with the antimicrobial peptide RiLK1. Therefore, it can be hypothesized that the immobilization process did not influence the folding of RiLK1 and electrostatic imbalances on the bacterial surface rather than a membrane permeabilization mode should be the main mechanism of antibacterial action of the bound peptide, as it is far too short to penetrate the cell membrane and create a hole.

Conclusion

Infectious, water-related diseases, many of which are caused by pathogens, represent a major reason for morbidity and mortality worldwide [23]. Therefore, the development of new strategies aimed at improving and maintaining the microbiology quality of drinking water is the crucial purpose of most beverage industries around the world. In this study, the previously characterized decapeptide RiLK1 was covalently grafted by the free amine groups to a PLA-straw surface, pre-activated with the plasma technique, with success and without using a spacer. Our results showed that immobilized RiLK1 maintained its antimicrobial activity and was highly efficacious at preventing bacterial growth against three of the most pathogen strains, relevant in the context of contaminated-water infections such as *E. coli*, *S. Typhimurium*, and *L. monocytogenes*. These results demonstrate that it is possible to ensure drinking water safety, preventing waterborne diseases by using functionalized drinking straws with AMPs included in reusable water bottles [24]. To the best of our knowledge, this is the first study aimed at the development of an innovative tubular device showing bactericidal activity, which can offer important advantages for a lot of applications in the beverage industry.

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2020.32.005219

G Palmieri, A Cammarano. Biomed J Sci & Tech Res

Submission Link: https://biomedres.us/submit-manuscript.php