Distinct Antimicrobial Analysis to Evaluate Multi-Component Wound Dressing Performance

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Abstract. Wound infection hinders adequate healing, being particularly grievous and prevalent in burn wounds and chronic wounds. Wound infection extends inflammation, preventing epithelialization and angiogenesis. Therefore, infection prolongs healing time, steeply increases treatment costs and degrades patients wellbeing. One successful strategy to control wound infection is to apply an active wound dressing, able to eliminate or significantly reduce the microbial population present at the infection site. Silver nanoparticles (AgNPs) are a multipurpose antimicrobial agent with a wide scope of applications which include wound dressings. Nevertheless, several studies denote AgNPs dose-dependent cytotoxicity, and their capability to bypass the blood-brain barrier and induce a neurotoxic effect. Hence, we propose to adopt two different strategies to attempt the simultaneously immobilize and increase the load of AgNPs within the wound dressing fabric. Thus, the envisaged objective is to prevent potential systemic cytotoxicity /through immobilization and to improve its antimicrobial capability due to the higher concentration of AgNPs. Two different approaches were used: i. AgNPs were suspended in an alginate (ALG) solution, ii. AgNPs were embedded in Mordenite (MOR) zeolite, followed by the addition of an ALG solution. Both suspensions were incorporated into polyester fabric assisted by its surface activation by dielectric barrier discharge (DBD) plasma treatment. The bactericidal and virucidal effectiveness of each composite was tested against bacteria species known to induce nosocomial infections and a bacteriophage that is a potential surrogate of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Two distinct antimicrobial analyses were used to provide insights on the antimicrobial effectiveness of the obtained composites and to indirectly assess the release of AgNPs.

Introduction

Virtually all human beings have experienced an event that led to a formation of a wound. In brief, wounds may occur due to trauma, cuts, incisions, abrasions, lacerations, punctures, burns, chemical corrosion and by medical interventions such as surgery [1]. Independently of the wound typology and its origin, a ubiquitous event for all wounds is the disruption of the integrity of the epidermis, the outer barrier of the skin. Epidermis represents a critical frontline against infection, and the wound healing process is focused on its prompt re-establishment. Immediately after epidermis disruption, vasoconstriction and fibrin clot is formed. Nevertheless, the wound site may rapidly become a problematic site of infection. Thus, the host immune system defenses are immediately triggered by the release of histamine, prostaglandins and bradykinin by mastocytes and basophils present at the injured site. These chemical signals enhance the permeation of blood granulocytes, particularly

neutrophils and complement proteins. Moreover, the release of these pro-inflammatory cytokines, chemokines and growth factors increase vascular permeation leading to other characteristic signs of acute inflammation such as redness, swelling, and heat. In a short time frame the wound site is populated with monocytes that mature into inflammatory macrophages, neutrophils and lymphocytes [2, 3]. This immediate response strategy successfully prevents the proliferation of microbial infection at the wound site, independently of pathogen type present, fungi, bacteria or virus. Therefore, the host system can focus on the wound healing process. In fact, macrophages play two additional important roles in wound healing. First by sweeping apoptotic cells, thus reducing the concentration of pro-inflammatory agents leading to the resolution of the inflammatory phase, and secondly by altering their phenotype, entering a reparative mode promoting angiogenesis by stimulating fibroblasts and Langerhans cell, endothelial cells and keratinocytes [4]. Fibroblasts start synthesizing collagen, promoting the formation of granulation tissue at the wounded site. Furthermore, collagen III matures into collagen I as the extracellular matrix (ECM) regeneration occurs with the settling of elastins, and glycoprotein components which encompass proteoglycans, laminins and fibronectin [5]. Capillary blood vessels are reconstructed and tissue functions are restituted. Acute wound healing unfolds between 4 to 6 weeks [6]. However, if the host immune system fails to complete at least one of the healing process events, the wound may degenerate to a chronic state. Table 1 depicts factors that may result in chronic wound formation.

Table 1: Factors responsible for prolonging wound healing and greatly enhance the probability of developing a chronic wound (adapted from 10.1177/0022034509359125).

Exogenous factors	Endogenous factors
Infection	Comorbidities
	Obesity
	Age
	Gender
	Medications and treatments
	Addictions
	Nutrition
	Wound type and wound extent

When developing a wound dressing, one of the most evident targets is the prevention of infection. Wound infection may be caused by fungi, virus and bacteria; yet, the most prominent are biofilm-forming bacteria [7]. These bacteria, once structured in a biofilm (enveloped in an extracellular polymeric substance) display an increased resistance to both host immune defenses and medical treatments. *Staphylococcus aureus* is usually used as model Gram-positive bacterium. However, it is also infamous for being responsible for nosocomial infections and for being able to form troublesome biofilms at wound sites. In fact, *S. aureus* biofilm infections account for more than 90 % of chronic venous ulcers [8]. *Escherichia coli* does not possess a representative prevalence in chronic wounds. Still, it may be considered as the most studied model of Gram-negative bacteria. The presence of bacteria in wound sites will impede on the resolution of the inflammatory phase [9].

Due to the current COVID-19 pandemic, viral infections are adequately receiving more attention by the general community. In spite of not being usually directly associated to chronic wounds or chronic wound development, a wound may still represent an important gateway for viral particles that may cause grievous systemic infections [10, 11].

Polyester (PES) is widely used material, being biodurability on of the most notable features for its common use in medical dressing applications (Table 2).

Table 2: Examples of commercial polyester-based medical textiles.

Application	Textile architecture	Composition in PES [%]	Brand
Post-operative patch	Non-woven	100	Taicend Technology
Undercast padding bandage	Non-woven	100	PediHealth
Tubular bandage	Knitted	100	Medelast
Medical tape	Non-woven	< 100	DetectaPlast
Wound dressing	Non-woven	< 100	Vliwasoft
Gauze swab	Non-woven	30	Pharmaplast

PES is, however, susceptible to microbial colonization, and allows *per se* biofilm formation. Therefore, to diminish the probability of microbial infection and biofilm establishment in the wound, PES may be functionalized using biocidal agents. Silver possess a renowned antimicrobial activity, which is potentiated when applied as silver nanoparticles (AgNPs) [10, 12]. Nevertheless, there are important issues when applying nanoparticles in wound dressings, namely their propensity to cause systemic cytotoxicity allied to the ability to permeate through the blood brain barrier [13]. Thus a strong entrapment of these nanoparticles within the wound dressing would be an adequate approach. To achieve this purpose woven PES surface was pre-activated using an atmospheric dielectric barrier discharge (DBD) plasma, a highly efficient surface pre-treatment for coating with AgNPs for medical use [14]. Moreover, the nanoparticles were mixed within an alginate (ALG) matrix, which consists in a biopolymer extracted from brown seaweed [15]. ALG comprises two monomers, L-guluronic acid (G) and D-mannuronic acid (M), and this biopolymer promptly crosslinks in the presence of polycations [10.1016/j.ijbiomac.2020.09.046]. Finally, Mordenite (MOR) zeolite was also tested as potential carrier for AgNPs. Zeolites consist in aluminosilicate minerals that comprising microporous architectures which may harbor the AgNPs [17].

Thus, to test if the AgNPs will have a systemic or local effect, the antimicrobial activity of functionalized PES was tested using two approaches, a static with direct contact with the microorganism, and a dynamic to evaluate its activity in the vicinity of the functionalized PES.

Materials and Methods

AgNPs synthesis. The AgNPs were synthesized according to the method described by Wan et al. [18]. Briefly, a starter seed solution was prepared by mixing and heating for 15 min at 70 °C 20 mL of citrate solution 1 % (w/v) and 75 mL of distilled water. Subsequently, 1.7 mL of a silver nitrate solution 1 % (w/v) was added, followed by a rapid addition of 2 mL of 0.1 % (w/v) sodium borohydride solution (freshly prepared). The final volume was adjusted using distilled water. The reaction was marinated at 70 °C for 1 h under vigorous stirring. The synthesis the AgNPs was then performed by boiling a mixture of 2 mL of 1 % (w/v) citrate solution and 80 mL of distilled water for 15 min. Then, 10 mL starter seeds were added under vigorous stirring, followed by 1.7 mL of 1 % (w/v) of silver nitrate. The reaction was maintained at 70 °C for 1 h.

AgNPs characterization. The size of the nanoparticles was estimated using an ultra-high resolution field-emission scanning electron microscope NanoSEM Nova 200 as described by Botelho et al. [19]. The AgNPs suspension was inserted in copper grids with carbon film 400 meshes, 3 mm diameter and using an electron acceleration of 18 kV. The mean size of AgNPs was extrapolated by measuring the average diameter of 250 nanoparticles using ImageJ software 1.53e [20]. The concentration of AgNPs was estimated by determining the silver content through atomic absorption spectroscopy (AAS) using a novAA(R) 350 AJ Analytical Instrumentation, after acid digestion.

DBD plasma treatment. Plasma application followed the same methodology as Zille et al. [14]. In summary, a semi-industrial DBD plasma prototype (Softal Electronics GmbH/University of Minho) was used at room temperature and atmospheric pressure. DBD plasma electrodes possess 50

cm effective width and a gap distance fixed at 3 mm. Its discharges consisted in 10 kV and 40 kHz. Each side of PES underwent 5 passages at 4 m min^{-1} and subjected to 1 kW power. Therefore, PES was submitted to 2.5 kW min m^{-2} of DBD plasma treatment. The effectiveness of DBD plasma was evaluated by measuring the water contact angle using sessile drop methodology as described by Padrão et al. [21]. Briefly, ultrapure water (3 μL droplets) were tested at room temperature using Data Physics OCA 15 equipment. Ten measurements were performed in distinct locations of PES, and the average contact angle was estimated.

PES functionalization. Immediately after DBD plasma treatment, the PES was dipped in a freshly prepared solution containing ALG 1 % (w/v), AgNPs 3.75x10^{-3} % (w/v) (herein named as ALG+AgNPs), or ALG 1 % (w/v), AgNPs 3.75x10^{-3} % (w/v) and Mordenite 2.5 % (w/v) (herein denominated as ALG+AgNPs+MOR). After dipping, PES was subjected to 5 passages through a padder (Roaches Padder BHP), 2.0 bar at 2 m min^{-1}. Afterwards, the functionalized PES (PES+ALG+AgNPs or PES+ALG+AgNPs+MOR) was immersed in a calcium chloride 2 % (w/v) aqueous solution for 5 min at 120 rpm of shaking speed to crosslink the alginate matrix. In the end, the functionalized PES was rinsed with distilled water to remove the excess of calcium chloride and was dried at 40 °C until constant weight was reached. The functionalization procedure was performed at room temperature.

Antimicrobial assays. The antimicrobial analyses were performed using *S. aureus* American Type Culture Collection (ATCC) 6538, *E. coli* ATCC 25922, and bacteriophage MS2 ATCC 15597B1.

To test the antimicrobial efficacy of the fabric at its surface as well as its vicinity, a "shake flask" methodology was applied following American Society for Testing and Materials (ASTM)-E2149-01 standard for bacteria and an adaptation of International Organization for Standardization (ISO) 18184 for the virus. Briefly, a pre-inoculum of each bacteria was prepared in tryptic soy broth (TSB) and incubated for 12 h at 37 °C and 120 rpm. Afterwards, each bacterium was centrifuged at 3900 *g*, 24 °C for 10 min. The supernatant was discarded and each bacterium was washed in a modified sterile phosphate buffer saline (PBS) (pH 7.4) and submitted to another centrifugation cycle. Subsequently, each bacterium concentration was adjusted to approximately 1 x 10^5 colony forming units per milliliter (CFU mL^{-1}) in PBS. 2 cm^2 fabric was aseptically immersed in PBS containing the bacterium and incubated for 5 h at 37 °C and 120 rpm. Then, each bacterium from each sample was collected, subjected to serial dilutions and incubated in tryptic soy agar (TSA) for approximately 14 h at 37 °C. Afterwards, the colonies were counted and the CFU mL^{-1} was determined. The anti-viral efficacy was estimated following an adaptation of the ISO18184. In short, 2 cm^2 fabric was immersed in ATCC medium 271 comprising approximately 1 x 10^5 plate forming units per milliliter (PFU mL^{-1}) of MS2, and incubated for 5 h at 37 °C and 120 rpm. Afterwards, each sample solution underwent a serial dilution and was inoculated in ATCC 271 solid medium freshly inoculated with *E. coli* ATCC 15597 (the host of MS2 bacteriophage). The Petri dishes were incubated for nearly 14 h at 37 °C. Then, the PFU were quantified and the PFU mL^{-1} was estimated.

To analyze the *in loco* antimicrobial activity, a "contact" methodology was performed using an adaptation of the American Association of Textile Chemists and Colorists (AATCC) TM 100 and ISO 18184. Fabrics with 6.25 cm^2 of surface area were inoculated with *S. aureus* ATCC 6538 or E. coli ATCC 25922, or MS2 bacteriophage ATCC 15597-B1. The concentration of each inoculum was 1x10^7 CFU mL^{-1} for the bacteria and 1x10^7 PFU mL^{-1} for the virus, using volume that covered the surface of the fabric but did not permeate or overflowed through it. After 1 hour of incubation at 24 °C, the fabrics were immersed in a solution of 100 fold the volume of the inoculum and were thoroughly vortexed during at least 1 minute. Each solution underwent a serial dilution and were inoculated in TSB (bacterium) or for MS2 solid medium 271 (ATCC) freshly inoculated with *E. coli* ATCC 15597 from a pre-inoculum with approximately 8 h of incubation 24 h at 37 °C and 120 rpm. The Petri dishes were then incubated for at least 14 hours at 37 °C and the PFU concentration was determined.

Results and Discussion

Fig. 1, depicts the synthesized AgNPs, which displayed a size of 15.91 ± 3.93 nm. AgNPs size has been reported as a highly important parameter for their antimicrobial activity. In addition, AgNPs have displayed an effective antibacterial activity within a wide range of sizes, from 8 nm to 40 nm [22]. Textile degree of hydrophilicity is paramount for interaction with compounds dissolved or suspended in water. Water is an easily available, environmental friendly, remarkable solvent. Plasma treatment is known to enhance hydrophilicity, thus improving fibres accessibility without compromising the textiles overall properties [23]. The DBD plasma treatment efficacy on PES is clearly shown in Fig. 2, by reducing the water contact angle to a value lower than 20°. Literature correlates this considerable reduction of water contact angle with a strong presence of oxygen-based functional groups generated by plasma [23].

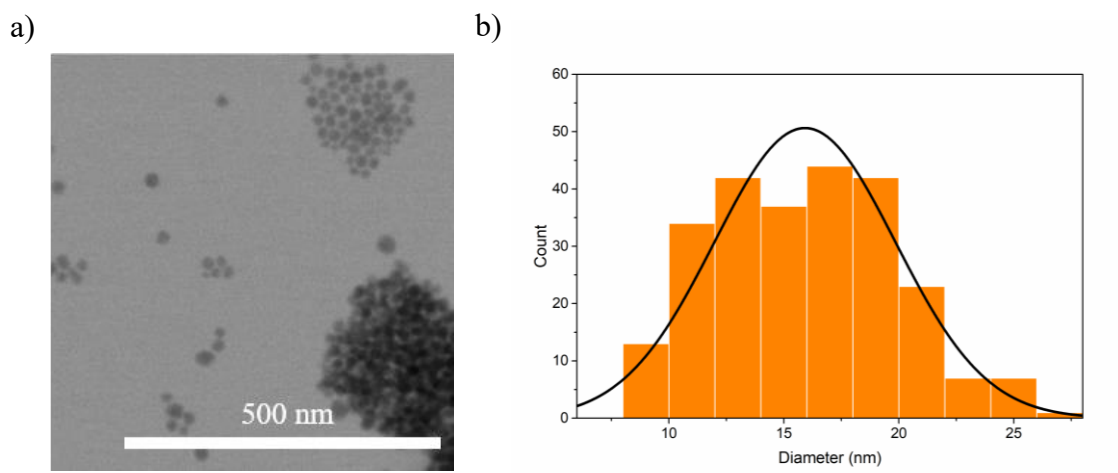a)   b)

Fig. 1: a) AgNPs STEM image and b) histogram depicting the AgNPs size polydispersity, the inset line describes the normal distribution.
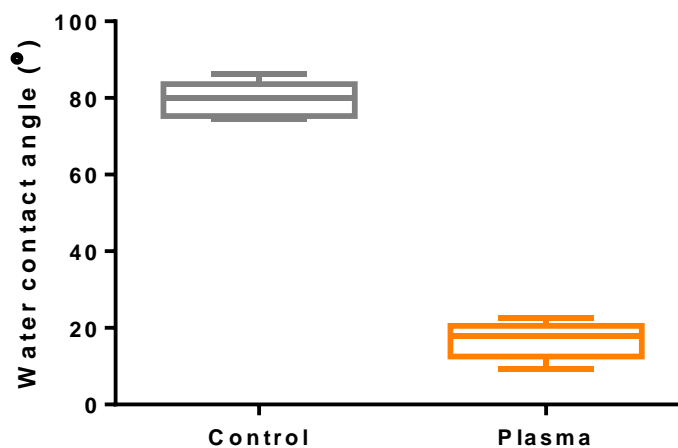

Fig. 2: Water contact angle of PES and PES treated with DBD plasma.

The antimicrobial properties of the functionalized PES were tested in two distinct settings, static ("contact") and dynamic ("shake flask"), to distinguish vicinity and *in loco* efficacy, respectively. Fig. 3 displays the antimicrobial properties of PES+ALG+AgNPs and PES+ALG+AgNPs+MOR against *S. aureus*, *E. coli* and MS2 virus.
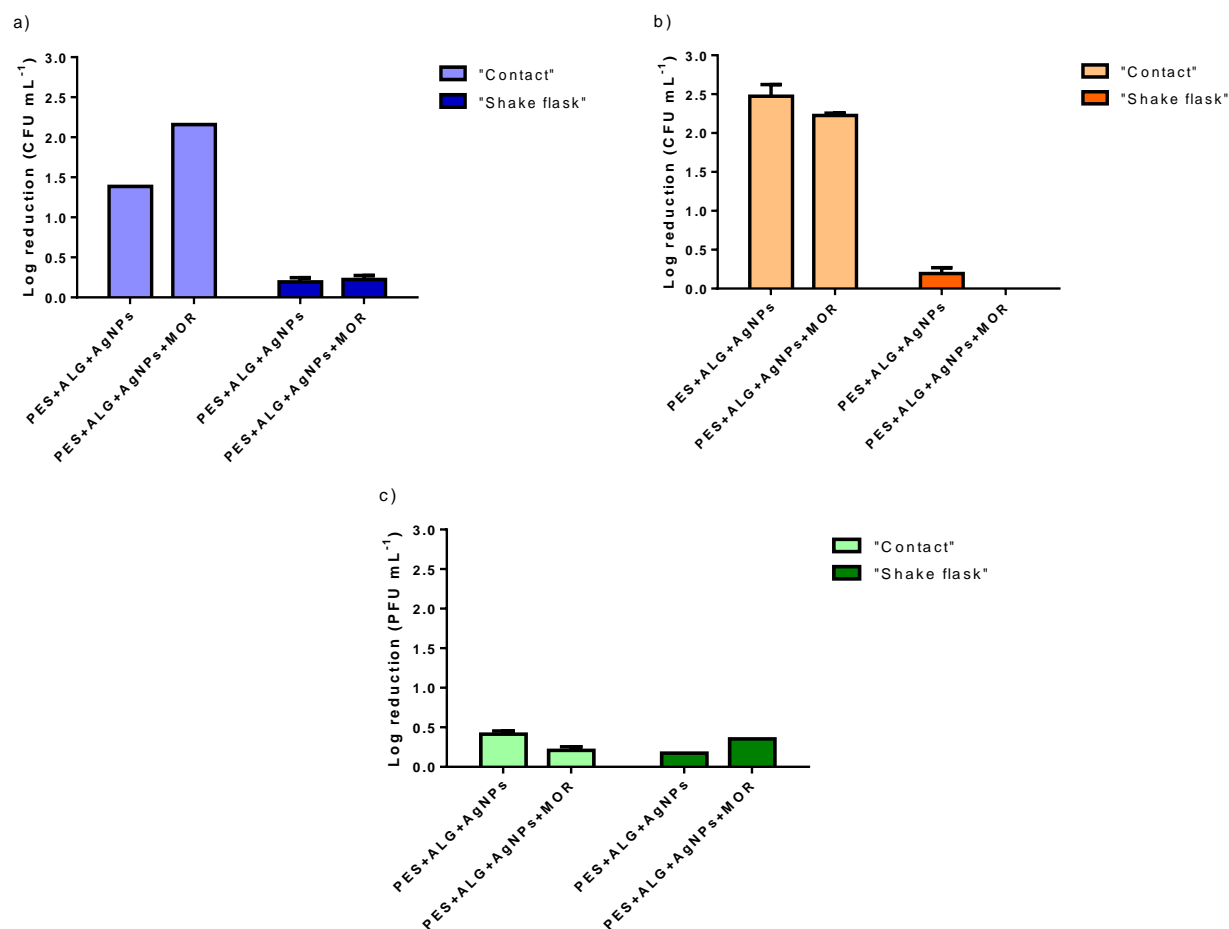
Fig. 3: Antimicrobial log reduction of a) *S. aureus*, b) *E. coli* and c) MS2. All tested samples were subjected to DBD plasma treatment, as non-plasma treated sample did not exhibit any activity (data not shown).

The bactericidal activity of PES+ALG+AgNPs against *S. aureus* may be considered as mild, reaching nearly 90 % of reduction in direct "contact" with the functionalized textile. Interestingly, for the same sample, the reduction was negligible (< 90 %) in dynamic conditions ("shake flask"). PES+ALG+MOR exhibited a higher bactericidal activity against *S. aureus* (approximately 99 %), probably due to the higher AgNPs entrapped within MOR microporous structure. Nevertheless, its activity was also very weak at the "shake flask" assay. *E. coli* assays displayed a similar reduction in the "contact" approach (approximately 99 %). Whereas in "shake flask" almost no reduction was observed for both PES+ALG+AgNPs and PES+ALG+AgNPs+MOR. Finally, the functionalized textiles did not show any relevant virucidal activity. These results infer that the use of DBD plasma treatment and ALG as matrix may effectively entrap the majority of the AgNPs in the PES woven fabric without hindering its direct bactericidal activity. If the AgNPs were released from the fabric, they would be homogenized in the solution and would exert a "systemic" bactericidal activity against the bacterium present in the "shake flask" solution. Therefore, this composite exhibits a potential strategy to be applied in a wound dressing that has the potential to kill the bacteria by direct contact, and simultaneously reduce the risk of systemic cytotoxicity of the AgNPs. Finally, MS2 was not vulnerable to AgNPs virucidal activity, as reported by You et al. [24]. This denotes the highly distinct behavior of compounds against bacteria and virus, underscoring the perils of generalization. Finally, it is advisable that the antimicrobial analysis should encompass both scenarios, antimicrobial effectiveness at the surface of the functionalized textile (contact) and at its vicinity (release). A textile designed to release an antimicrobial agent may or may not have activity at its surface (contact). On the other hand, a textile functionalized to solely eliminate microorganisms at its surface may have an

activity at its vicinity due to unwanted release of the antimicrobial agent, or by generating other species (such as reactive oxygen species). Without both analysis, it is not possible thoroughly describe the antimicrobial processes involved.

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