SYNERGISTIC ACTIVITY OF A NOVEL CLASS OF AZOIMIDAZOLE DYES WITH POLYVINYLPYRROLIDONE-SILVER NANOPARTICLES FOR THE DEVELOPMENT OF ANTIBACTERIAL TEXTILES

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ABSTRACT

The high demand for novel antimicrobial textiles by the medical, health care, hygiene, sportswear, personal protective equipment, and filtration sectors promoted the growth of functional textiles. However, the efficacy of antimicrobial agents against different pathogens is a considerable challenge due to the distinctive mechanisms of action and resistance. The development of novel synergistic antimicrobial agents may offer numerous opportunities to enhance antimicrobial effectiveness, namely boost the activity of individual agents, reduce dosages, minimize toxicity, and amplify the activity spectrum. On the one hand, azo dyes containing a heterocycle present good tinctorial strength and brightness of shades. In particular, the imidazole ring also has interesting antimicrobial, analgesic, and anti-inflammatory properties. On the other hand, silver nanoparticles (AgNPs) are renowned antimicrobial agents against a wide range of microorganisms, but their application is limited by the toxicity observed for effective concentrations. In this work, a novel class of azoimidazoles (AzoIz) and corresponding precursors (AmIz) were conjugated with polyvinylpyrrolidone-coated AgNPs, and their synergistic effect was assessed against *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa*. The results showed interesting antimicrobial properties of the novel AzoIz molecules when combined with a very small concentration of AgNPs. Thus, the application of these conjugates in textiles may lead to highly colored materials with remarkable antimicrobial properties, which worth to be further explored.

Keywords: synergism, nanoparticles, amidrazones, azo compound, imidazole, antibacterial, textiles

1. INTRODUCTION

Antimicrobial textiles are functional materials, which can kill the microorganisms or inhibit their growth, and are extremely useful to prevent the degradation of the materials, avoid cross-contamination, or even treat microbial infections [1,2]. Antimicrobial agents applied in textile materials should present low toxicity to consumers, be effective against a large spectrum of pathogens and be selective only to unwanted microorganisms [2]. The high demand for novel antimicrobial textiles by the medical, health care, hygiene, sportswear, personal protective, and filtration sectors increased the interest in functional textiles [3].

The antimicrobial compounds and their safety have been a subject of constant research. Different types of antimicrobial textiles have been developed containing organo-metallics, phenols, triclosan, quaternary ammonium compounds, inorganic nanoparticles, and antibiotics [4-6]. Moreover, functional dyes, which can have an antimicrobial character, have been a strategy to combine both functional finishes and conventional textile dyeing into one process [7]. However, the efficacy against different pathogens is a considerable challenge due to the distinctive mechanisms of action and resistance. The development of novel synergistic antimicrobial agents may offer numerous opportunities to enhance antimicrobial effectiveness, such as boosting the activity of individual agents, reducing dosages, minimizing toxicity, and amplifying the activity spectrum [8]. Synergistic approaches combine at least two substances that result in a superior efficacy when compared to that of the individual agents [9,10].

Azobenzene molecules are the most used scaffold for the development of colored materials and the ones containing a heterocycle present good tinctorial strength and brightness of shades [11,12]. Furthermore, non-commercial azobenzenes holding heteroaromatic moieties can also lead to interesting antimicrobial properties [13]. In particular, the imidazole ring showed to have antimicrobial, analgesic, and anti-inflammatory properties [14].

Nanomaterials may offer innovative routes to address the development of advanced products by improving some materials' properties or implementing new ones. Inorganic nanoparticles have been widely studied due to their unique characteristics: the small size and high surface-volume ratio, ability to act at the cellular level, improved solubility, surface adaptability, and multifunctionality [8,15]. Silver nanoparticles (AgNPs) are renowned antimicrobial agents against a wide range of microorganisms including bacteria, fungi, and viruses with multiple mechanisms of action [16,17]. However, their application is limited due to concerns about their potential hazards to the environment and health. Their properties and corresponding toxicity profile are complex and may undergo numerous dose-, size- and time-dependent mechanisms. For these reasons, the safe-by-design concept gains exponential importance once it foresees the risk assessment in the early stages of development [18,19].

In this work, a novel class of azoimidazole (AzoIz) dyes and corresponding precursors (amidrazone-derivatives containing an imidazole ring (AmIz)) were conjugated with polyvinylpyrrolidone-coated AgNPs. The AmIz and AzoIz were characterized by proton nuclear magnetic resonance (^{1}H NMR) and Fourier-transform infrared spectroscopy (FTIR). The color of the compounds in an aqueous solution was evaluated by ultraviolet-visible (UV-Vis) spectrophotometry. The synergistic effect of the conjugates was assessed against *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa.* The application of the AzoIz in textiles may lead to colored materials and their conjugation with AgNps may produce remarkable antimicrobial properties, which worth to be further explored in future research works.

2. MATERIALS AND METHODS

2.1. Materials

Commercial polyvinylpyrrolidone-coated AgNPs (20-30 nm) were obtained from SkySpring Nanomaterials Inc. All the other reagents were purchased from Sigma-Aldrich without any purification. The reactions were followed by thin-layer chromatography (TLC) using Macherey-Nagel™ aluminum sheets UV254. The compounds were characterized by ^{1}H NMR and FTIR. The NMR spectra were performed at room temperature on a Bruker Avance 3400 (^{1}H: 400 MHz). The data were reported by chemical shifts (ppm), multiplicity (s -

singlet, brs - broad singlet d - doublet, t - triplet, dd - doublet of doublets, or m - multiplet), and integration. The IR spectra were obtained using KBr cells on a Shimadzu IR-Affinity 1 FTIR spectrophotometer with an attenuated total reflectance accessory (ATR-Dia/KRS-5). UV-Vis spectrophotometry (Shimadzu, UV-1800) was used to measure the absorbance spectra of the compounds in water with a standard quartz cuvette.

2.2. Synthesis of the AmIz compounds

5-amino-4-(cyanoformimidoyl)-1*H*-imidazoles were the initial compounds used in this work and were obtained from commercial reagents including diaminomaleonitrile (DAMN), triethyl orthoformate (TEOF), and *p*-anisidine or methylamine according to a previous method developed by Alves *et al* [20]. Then, the corresponding amidrazones were prepared according to a previous method developed by the research group with slight differences described above [21].

2.2.1. (*Z*)-5-amino-1-(4-methoxyphenyl)-*N*′-phenyl-1*H*-imidazole-4-carbohydrazonamide (AmIz-a)

To a 25 mL flask containing 2.0 g of 5-amino-1-(4-methoxyphenyl)-1*H*-imidazole-4-carbimidoyl cyanide (8.3 mmol) and 10 μL of acetic acid in ethanol (8 mL) was added 1.25 mL of phenylhydrazine (12.5 mmol), under nitrogen atmosphere. The suspension was placed under magnetic stirring at 8 °C for 17 hours. The compound was obtained in 94% yield (2.5 g; 7.8 mmol).

2.2.2. *(Z)*-5-amino-1-methyl-*N*′-phenyl-1*H*-imidazole-4-carbohydrazonamide (AmIz-b)

To a 25 mL flask containing 5-amino-1-methyl-1*H*-imidazole-4-carbimidoyl cyanide (1.1 g, 7.0 mmol) and 10 μL of acetic acid in ethanol (3 ml) was added 1.25 mL phenylhydrazine (10.5 mmol), under nitrogen atmosphere. The suspension was placed under magnetic stirring at 8°C for 3h. The compound was obtained pure by filtration in 96% (1.6 g, 6.8 mmol)

2.3. Synthesis of the AzoIz compounds

The 5-amino-*N*′-phenyl-1*H*-imidazole-4-carbohydrazonamides were used to prepare azoimidazoles. Briefly, silver nitrate (3.0 molar equiv) was dissolved in acetonitrile (2.5 mL) in a 50 mL flask. Then, the amidrazones were suspended in an acetonitrile/ethanol mixture (80:20) (10 mL), and the suspension was added to the silver nitrate solution under magnetic stirring at room temperature. A dark red suspension was formed immediately and, after 15 minutes, the solid was filtered and washed with acetonitrile and ethyl ether. The metallic silver formed during the synthesis was removed by centrifugation.

2.4. AgNPs redispersion, and preparation of the solutions containing the AgNPs and the AmIz or AzoIz compounds

First, AgNPs were redispersed in water with a concentration of 10 $\mu g.mL^{-1}$ using an ultrasonic bath for 30 minutes and an ultrasound tip for more than 30 minutes. Then, water solutions containing 256 $\mu g.mL^{-1}$ of each organic compound were prepared. To the solutions containing AmIz compounds, nitric acid (2.0 molar equiv) was added to obtain the solubility in water. The AgNPs dispersion and organic compound solutions were mixed (1:1) (1.5 mL) and the dispersion was kept in an ultrasound bath for 15 minutes.

2.5. Antimicrobial evaluation

The antimicrobial properties were assessed by determining the minimal inhibitory concentration (MIC) based on the methodology described by Wiegand *et al.* [22]. The evaluated microorganisms were *S. aureus* American Type Culture Collection (ATCC) 6538, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. Briefly, a pre-inoculum of each bacterium was prepared using tryptic soy broth (TSB) for *S. aureus* and *E. coli* or in nutrient broth (NB) for *P. aeruginosa*. All pre-inocula were incubated overnight at 37 °C with a shaking speed of 120 rpm. Each one of the synthesized compounds was subjected to a serial dilution in water (Table 1). Subsequently, each dilution was diluted 50 % (v/v) of inoculated with $1x10^7$ CFU.mL^{-1} of each bacterium in its corresponding fresh culture medium in flat-bottom 96-well plates. In the positive control samples, the bacteria suspensions were diluted in water without any compounds. The optical density (OD, wavelength: 600 nm) of each bacterium suspension was immediately measured using a microplate reader 96-well plates. Afterward, the 96-well plates were incubated for approximately 20 hours at a shaking speed of 120 rpm and 37 °C. The OD was determined, and the MIC was defined visually using a magnifying glass. The MIC was attributed to the lower dilution without turbidity. Furthermore, the MIC was confirmed using equation 1. Each dilution was replicated twice.

$$OD = OD_f - OD_i \quad \textbf{Equation 1}$$

Where OD corresponds to the optical density (OD) of each sample, OD_i to the initial OD, and OD_f to the OD measured after 20 hours of incubation.

Table 1. Concentrations of the initial solution (IS) and corresponding dilutions (D1-6) (μg.mL^{-1}) of the azoimidazoles (AzoIz), corresponding amidrazone precursors (AmIz) and silver nanoparticles (AgNPs) that were used for synergistic studies and minimal inhibitory concentration calculation.

	IS	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
AgNPs	10	5	2.5	1.3	0.6	0.3	0.2
AmIz or AzoIz	256	128	64	32	16	8	4

3. RESULTS AND DISCUSSION

5-amino-*N′*-phenyl-1*H*-imidazole-4-carbohydrazonamides (AmIz) was previously synthesized by our research group using a mild and simple method. The synthesis started from the accessible 5-amino-4-cyanoformimidoyl imidazoles. The obtained AmIz showed interesting antimicrobial activity against *Candida krusei*, *Candida albicans,* and *Cryptococcus neoformans*, and also against biofilm formation. However, their activities against filamentous fungi (*Trichophyton rubru*m, *Trichophyton mentagrophytes*, *Microsporum gypseum*) and bacteria (*S. aureus, E. coli,* and *P. aeruginosa*) were very weak when compared to the activity on yeasts [21,23,24]. Moreover, it was noticed that these compounds readily oxidized when exposed to the air by the emergence of colored solids and solutions. Thus, the reactivity of these compounds using silver nitrate as an oxidizing agent was tested. This reaction immediately occurs giving the azo molecules a strong red color (Figure 1). Considering the above-mentioned properties, in this work, the conjugation of these compounds (AmIz and AzoIz) with AgNPs was studied to obtain dyes with high coloration strength and improved antimicrobial properties. The AmIz or AzoIz compounds were conjugated in concentrations above their MIC found for the agents individually against *S. aureus*, *E. coli,* and *P. aeruginosa*. The MIC for AgNPs alone was found to be from 1250

to 4000 μg.mL^{-1} and for organic compounds from 32 to >256 μg.mL^{-1}.[25] The conjugates were obtained by ultrasound mixing a dispersion of AgNPs (10 μg.mL^{-1}) and organic compounds solutions (256 μg.mL^{-1}) (Figure 1).

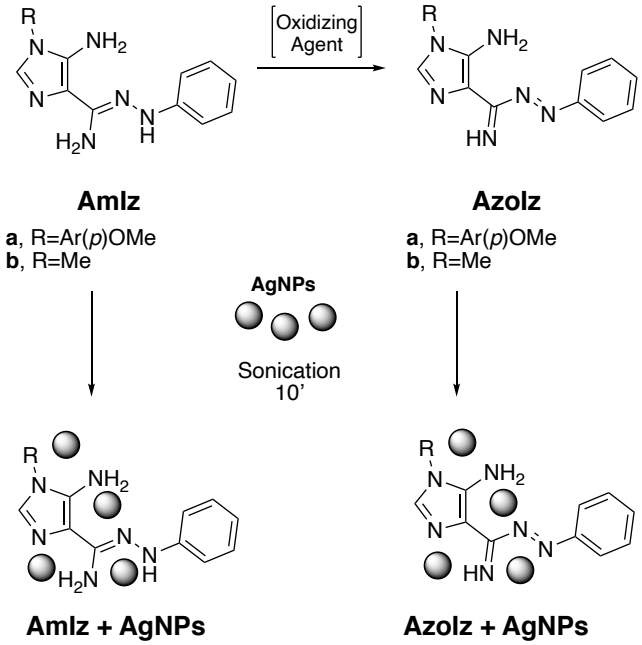

Figure 1. The methodology adopted to obtain the organic compounds (amidrazones (AmIz) and azoimidazoles (AzoIz)) conjugated with silver nanoparticles (AgNPs).

The AmIz and AzoIz compounds were characterized by UV-Vis, FTIR and ^{1}H RMN spectroscopies to verify the occurrence of the oxidation reaction. The FTIR spectra showed the evident appearance of two bands in the region of C=N bonds (at 1678 and 1639 cm^{-1} for AzoIz-a; at 1670 and 1639 cm^{-1} for AzoIz-b) that supports the conversion of the hydrazone to azo group (Figure 2). Moreover, in the ^{1}H RMN, it was possible to detect the shift of the proton signal at δ 7.10 - 7.13 ppm from the imidazole 2-*H* proton in AmIz structure to δ 7.88 - 8.02 ppm in the AzoIz. All the other proton signals also shifted to higher chemical shifts due to the withdrawing effects of the positive charge on the imidazole ring (Figure 3-a). Supporting the emergence of the red color, the UV-Vis spectra showed the appearance of an intense absorbance band around 493 nm in the AzoIz structures. The solution of compounds varied from uncolored (AmIz) to red (AzoIz) (Figure 3).

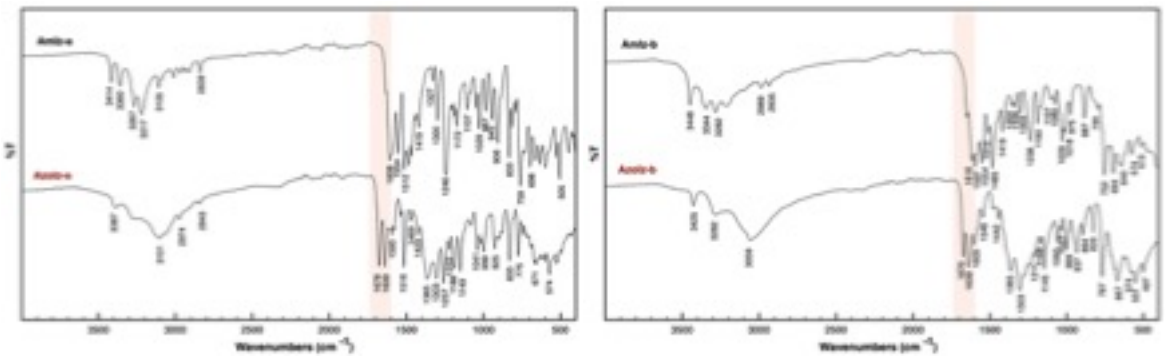

Figure 2. FTIR spectra of the compounds.

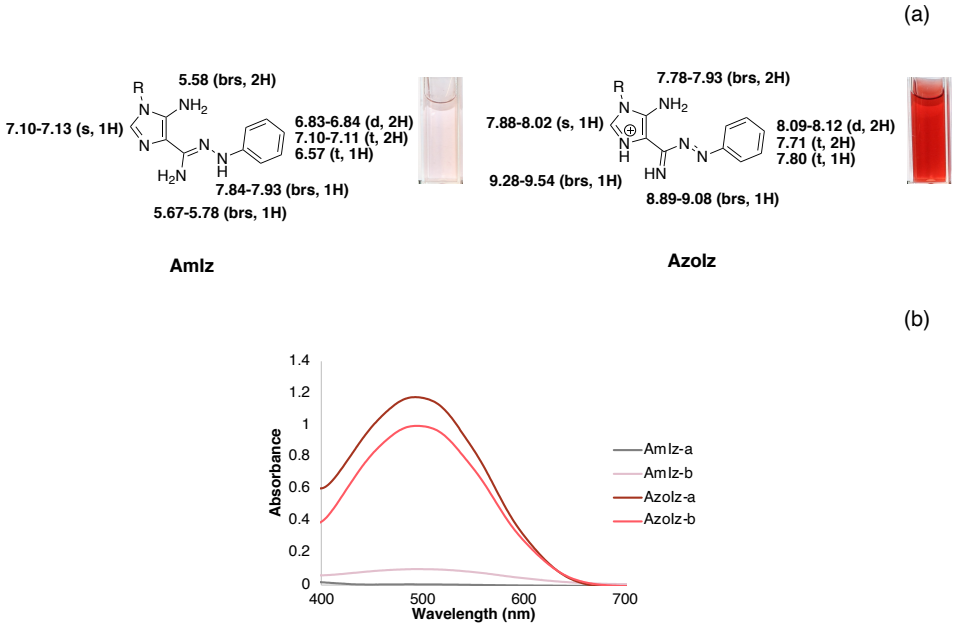

Figure 3. ^{1}H RMN spectra of the amidrazones (AmIz) and azoimidazoles (AzoIz) molecules and the emergence of red color in the AzoIz water solutions (a) UV-Vis spectra of the AmIz and AzoIz molecules (b).

The synergistic studies using AmIz and AzoIz molecules were assessed by MIC calculation (Table 2). Combining AgNPs with AmIz structures was not possible to obtain any additional antimicrobial effect against *S. aureus, E. coli,* and *P. aeruginosa* in the tested concentrations. On the opposite, interesting synergistic effects were observed using the AzoIz molecules, especially in Gram-negative bacteria (*E. coli* and *P. aeruginosa*). In compound AzoIz-a, the effective concentration decreased from >128 μg.mL^{-1} to 16 to 32 μg.mL^{-1} by the addition of a small concentration of AgNPs (0.6 - 1.3 μg.mL^{-1}). In the compound AzoIz-b, the concentration decreased from >128 μg.mL^{-1} to 4.0 - 32 μg.mL^{-1} by the addition of AgNPs (0.2 - 1.3 μg.mL^{-1}) (Table 2). The inferior antibacterial action against *S. aureus* can be justified by the differences in the structure of the cell walls of the two types of bacteria. *S. aureus* presents a thicker peptidoglycan layer (30 nm thickness) comparing to the thinner structure of the *E. coli* cell wall (~3–4 nm thickness). The positively charged AgNPs and AzoIz compounds may be easily retained in the negative and thicker peptidoglycan layer of *S. aureus* bacteria.

Table 2: Synergistic antibacterial activity of the amidrazones (AmIz) and azoimidazoles (AzoIz) alone and respective conjugates with silver nanoparticles (AgNPs) (minimal inhibitory concentration (MIC, μg.mL^{-1})).

	AgNPs	**AmIz-a**	**AmIz-a +AgNPs**	**AmIz-b**	**AmIz-b +AgNPs**
S. aureus	> 5.0	≥128+≥5	≥128	32	≥128+≥5
E. coli	> 5.0	≥128+≥5	≥128	32	≥128+≥5
P. aeruginosa	> 5.0	≥128+≥5	≥128	32	≥128+≥5

	AgNPs	**AzoIz-a**	**AzoIz-a +AgNPs**	**AzoIz-b**	**AzoIz-b +AgNPs**
S. aureus	> 5.0	64	64+2.5	**>128**	**64+2.5**
E. coli	> 5.0	**>128**	**32+1.3**	**>128**	**32+1.3**
P. aeruginosa	> 5.0	**>128**	**16+0.6**	**>128**	**4+0.2**

4. CONCLUSIONS

This study reported the potential of the novel AzoIz molecules to be used both as an antimicrobial agent and as a dye for textile applications. Considering these results, synergistic effect between the AzoIz or AmIz molecules with AgNPs will be subsequently optimized by varying the concentrations. An interesting structure-activity relationship might result once the compound AzoIz-b, which contains a methoxyphenyl group, showed improved results. The antimicrobial mechanism of action, the antimicrobial action against other pathogens (e.g. fungi), the cytotoxicity tests, and the textile dyeability process will be also an object of analysis.

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