Enhanced photoinduced antibacterial activity of a BODIPY photosensitizer in the presence of polyamidoamines.

ABSTRACT

Photosensitizers belonging to the boron-dipyrromethenes (BODIPYs) class were recently found endowed with good efficacy in the antibacterial photodynamic therapy (aPDT) against both Gram-positive and Gram-negative bacteria. In this paper, we report on the remarkable adjuvant effect exerted in this respect by linear polyamidoamines (PAAs), a family of moderately basic polymers obtained by Michael-type polyaddition of amines to bisacrylamides. Three different PAAs (AGMA1, BP-AGMA and BP-DMEDA) were studied, testing for each two different molecular weight samples (8.000 and 24.000 Da). At non-toxic concentrations (1 or 10 μg mL^{-1}) all PAAs remarkably improved the killing efficacy of BODIPY upon irradiation with a green LED device (range: from 480 to 580 nm with λ_{max} = 525 nm) up to an energy rate of 16.6 J cm^{-2}. A 6 – 7 log units decrease in bacteria survival was observed with concentrations of BODIPY of 1.0 μM and 0.1 μM in the case of *Escherichia coli* and *Staphylococcus aureus*, respectively. The one-way analysis of variance (ANOVA) was used to evaluate the statistical significance of different treatments ($n \geq 3$). Thus, the PAA-photosensitizer combination warrants potential as a new, effective and mild method of killing bacteria. Moreover, the antibacterial treatment here reported might be successfully applied to defeat the bacterial resistance often encountered with many antibacterial drugs owing to the double action of this two-components treatment.

Keywords BODIPY · Polyamidoamine (PAA) · Antimicrobial Photodynamic therapy (aPDT) · Green LED

Introduction

The Photo-Dynamic chemo-Therapy (PDT) concerns the treatment of a localized pathology *via* the administration of a photosensitizer (PS), following irradiation of the diseased area with low energy electromagnetic field (i.e. visible light) [1].

In medicine, PDT is successfully applied for the treatment of several diseases such as localized cancers or precancerous malignancies, particularly those developed on the skin. However it finds application also in the treatment of localized infections occurring after cutaneous lesions arisen after burns or wounds or, even more, for the disinfection of dental channels [2].

Antibacterial PSs should exhibit one or more cationic groups on its frame, thus allowing an ionic interaction with the negatively charged bacterial outer envelope. To this aim, phenothiazines, cationic phthalocyanines, cationic porphyrins are the most frequently PSs reported in the literature [2, 3]. Together with the cationic moiety, it is known that the binding of PSs to microorganisms could be enhanced by the presence of a complementary moiety of the PS characterized by the presence of a neutral appendix penetrating the lipidic membrane; this effect is the so called "snorkel effect" [4].

In the last decade, a new class of dyes, the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacenes, entered the scenario of antibacterial PSs. These molecules, also known as boron-dipyrromethenes (BODIPYs), are easy synthesizable, versatile chromophores that have found application in different fields (mainly related to their fluorescent efficacy), such as DNA labelling, ion sensing, solar cells, and in light harvesting systems [5, 6]. BODIPYs could be used as photosensitizers in virtue of a structural modification aimed at inhibiting their strong fluorescence. This effect is generally obtained by the introduction of heavy atoms, such as iodine atoms, in the 2,6-pyrrole positions, as first reported by Nagano [7]. In previous works, we reported that a cationic BODIPY (2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4'-difluoroboradiazaindacene (BOD-NMe) (Figure 1A) showed an interesting antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* biofilm [8, 9]. This molecule is characterized by a positively charged, methyl pyridinium substituent, in the *meso* position of the BODIPY skeleton.

The focus of many studies on antibacterial photodynamic therapy is addressed to improve the PS interaction to microorganisms by means of liposomes [10], quaternized chitosan hydrogels [11], auto-assembly PS-nanoparticles [12] and poly(lactic-co-glycolic) (PLGA) nanoparticles [13].

The aim of this study was to verify the possible role of cationic polyamidoamines (PAAs) as adjuvants in antimicrobial PDT (aPDT). PAAs are *tert*-amino polymers obtained by polyaddition of amines to bisacrylamides, which can be designed to be biocompatible and biodegradable [14]. Microbiological assays have been performed in order to investigate if three PAAs, characterized by two different molecular weight (8000 and 24000 Da), increased the photoinactivation rate of the BODIPY (BOD-NMe).

Materials and Methods

Photosensitizer and polymers

2,6-Diiodo-1,3,5,7-tetramethyl-8-(N-benzyl-4-pyridyl)-4,4'-difluoroboradiazaindacene (BOD-N-Me) (University of Insubria, Varese, Italy), the photosensitizer administered to photoinactivate bacteria, was synthesized according to a previously reported synthetic procedure [8]. A stock 1 mM (water:acetone-1:1) (sterilize water with a Direct-Q 3 UV with Pump of Millipore, Vimodrone (MI) Italy; acetone analytical grade by Sigma Aldrich, Milano, Italy) by solution was prepared as previously described [8] and diluted in Phosphate Buffer (PB) at pH 7.4 constituted of KH_2PO_4/K_2HPO_4 (Sigma Aldrich, Milano, Italy) 10 mM to the final chosen concentration (0.1 or 1 μM).

The Polyamidoamines (PAAs) tested, as potential adjuvant in antimicrobial PDT, are BP-DMEDA, BP-AGMA and BP-DMEDA (University of Milan, Milan, Italy) and they were prepared according to a previously reported synthetic procedure [14]. They were fractionated by selective ultrafiltration on different membranes with cut-off, in the order, 100, 50, 20, 10 and 5 KDa (EMD Millipore Amicon™ Bioseparations Stirred Cells). For each sample, two fractions of average molecular weight 8000 and 24000 Da were selected for the biological tests. They corresponded

respectively, to the fraction retained between the 50- and 20 KDa and the fraction retained between 10 and 5 KDa membranes. The actual number-average molecular weights (M_n) were determined by SEC-LALS [14]. Water stock solutions of PAA (1 mg mL^{-1}) at two sizes, 8000 and 24000 Da, were administered to the cells at different final concentrations (1, 5 and 10 μg mL^{-1}).

Irradiation device

The green LED array is composed of 12×3 W diodes distributed on an 11 cm diameter disk, and equipped with a heat sinker. The emitted light is characterized by a lambda max (λ_{max}) at 525 nm (range: from 480 to 580 nm) (fluence rate 4.6 x 10^{-3} W cm^{-2}, light energy density, or fluence, 4.14, 8.28 and 16.56 J cm^{-2} for 15, 30 and 60 min of irradiation, respectively) and a width at half maximum of 50 nm. The electric supply was ensured by a 50 W current transformer. This array was placed above the plate at such a distance as to produce a homogeneous area of irradiation with a fluence rate of 7.52×10^{-5} W cm^{-2} at 525 nm (λ of maximum emission), as determined with a LI-1800 spectroradiometer (LI-COR, Lincoln, Nebraska, USA). The green LED array, the heat sinker and the 50 W current transformer were producted by Lomar Elettronica located in Flero (BS) Italy.

Microorganisms

Staphylococcus aureus ATCC 35033 was purchased from American Type Culture Collection (ATCC) (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy); *Escherichia coli* C1a [15] was obtained as a gift from University of Milan. They were chosen as Gram-positive and Gram-negative model microorganisms and were routinely grown in Luria-Bertani (LB) (Sigma Aldrich, Milano, Italy) broth or on LB Agar plates (Sigma Aldrich, Milano, Italy) under aerobic conditions at 37°C.

Photodynamic assays

In order to rule out dark toxicity of PAAs, overnight cultures of the model microorganisms were diluted with PB to achieve a cell concentration equal to 10^8 CFU mL^{-1}. The PAA solution was added to bacterial samples to obtain the chosen PAA concentration (1, 2.5, 5 or 10 μg ml^{-1}) for both sizes

polymer (8.000 Da and 24.000 Da). The untreated and PAA treated samples were dark incubated at 37 °C for 70 min and the cellular concentration was checked by a plate count technique and expressed as colony forming units (CFU mL^{-1}).

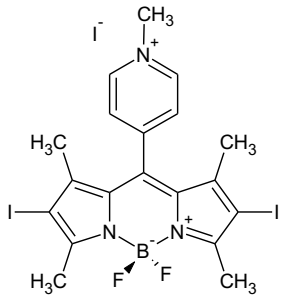
To induce photoinactivation, model microorganisms, grown overnight in LB and diluted to 10^8 CFU mL^{-1}, were treated with sub-optimal BOD-N-Me concentrations, 1 μM for *E. coli* and 0.1 μM for *S. aureus*. PAAs were administered at 1 μg mL^{-1} for *E. coli and* 10 μg mL^{-1} for *S. aureus*. Bacteria were incubated 10 min in the dark and irradiated 15, 30 and 60 min under green LED device. In each experiment, the following controls were set up: a sample exposed to the photosensitizer and not irradiated (+PS, –light), a sample without the photosensitizer and not irradiated (–PS, –light), a sample without the photosensitizer and irradiated (–PS, +light), a sample exposed to PAAs and not irradiated (+PAA, -light), a sample exposed to PAA and irradiated (+PAA, +light). The PDT effect on cellular viability was evaluated after irradiation by means of a plate count technique and expressed as colony forming units (CFU mL^{-1}).

Statistical analysis

The experiments were repeated at least three times on separate dates. Mean and SD calculations were performed using Microsoft Excel 2010. Data, normally distributed, were analysed by means of one-way ANOVA (Origin_7.0 SR0; Origin lab, Origin Lab Corporation Northampton, Massachusetts, USA). Significant treatment effects were estimated ($p < 0.05$ and $p < 0.01$, $0.94 < 1 - \beta < 1$).

Results

In the present PDT study, the improvement of the bacterial photoinduced inactivation exerted by the above cited BODIPY (Fig. 1A) in the presence of three different PAAs (Fig. 1B) has been evaluated.

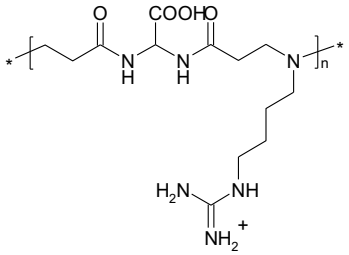


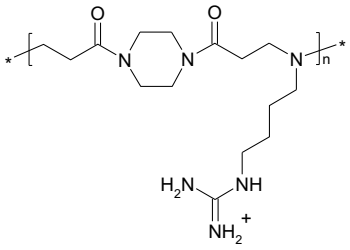
BOD-NMe

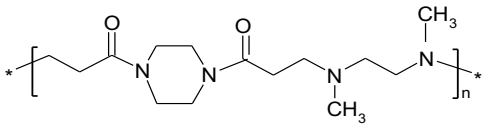
$C_{19}H_{19}BF_2I_3N_3$

Mw = 718.89

A







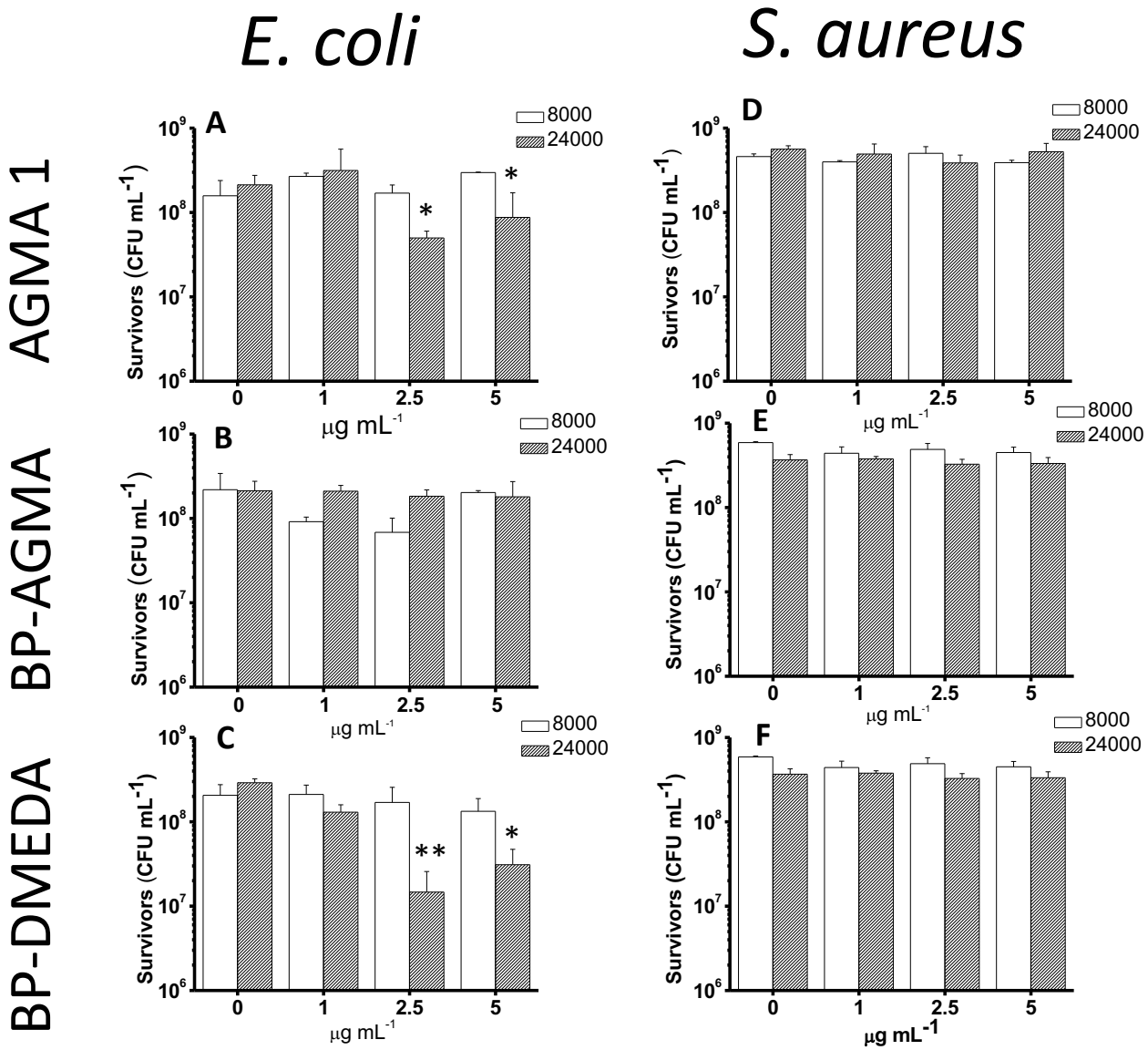
AGMA1	BP-AGMA	BP-DMEDA
Mw ~ 8000	Mw ~ 8000	Mw ~ 8000
(~ 12 positive charges)	(~ 40 positive charges)	(~ 30 positive charges)
Mw ~ 24000	Mw ~ 24000	Mw ~ 24000
(~ 36 positive charges)	(~ 120 positive charges)	(~ 90 positive charges)

B

Fig. 1 (A) Chemical structure, Molecular formula, and Molecular weight (Mw) of BOD-NMe. (B) Chemical structure of the repeating units of the three polymers with their molecular weight and the corresponding positive charges at pH = 7.2

In the present context, the ideal antibacterial activity adjuvant should be intrinsically non-toxic to bacterial cells and insensitive to light. The PAA intrinsic toxicity was evaluated in the dark on *Escherichia coli* and *Staphylococcus aureus*. *S. aureus* was not sensitive to dark treatment of PAAs 8000 and 24000 Da up to 10 μg mL^{-1}, while *E. coli* was slightly sensitive to 2.5 and 5 μg mL^{-1} AGMA-

1 and BP-DMEDA 24000 Da (Fig. 2). The slight decrease of one log unit of bacterial concentration
2 was statistically significant: AGMA-1 24000 Da 2.5 and 5 μg mL^{-1} $p = 0.049$ ($1 - \beta = 0.94$) and $p = 0.033$ ($1 - \beta = 0.98$), respectively; BP-DMEDA 24000 Da 2.5 and 5 μg mL^{-1} $p = 0.002$ ($1 - \beta = 1$)
3 and $p = 0.020$ ($1 - \beta = 0.99$), respectively.
4



5

6 **Fig. 2** Effect of PAAs on *Escherichia coli* and *Staphylococcus aureus*. Bacteria were treated for 70
7 min in the dark with increasing concentrations of cationic polymers with different Molecular Weight
8 (Average Molecular Weight 8000 and 24000 Da). The bars represent bacterial concentrations
9 expressed as CFU mL^{-1} of three biological replicates, whereas error bars represent the standard
10 deviation from the mean, ** $p \leq 0.01$, * $p \leq 0.05$ (one-way ANOVA)

To photoinactivate bacteria the PS and PAA concentrations were established according to the sensitivity of the bacterial strain to both chemicals. Based on previous data [8], the experiments on *E. coli* and *S. aureus* were carried out using 1.0 μM and 0.1 μM BOD-NMe concentrations, respectively. These PS concentrations induced a decrease of 3 – 4 log units of a 10^8 CFU mL^{-1} bacterial samples after 60 min irradiation with the green LED. The non-toxic concentrations of the PAAs used in the photodynamic experiments, were 1.0 μg mL^{-1} for *E. coli* and 10 μg mL^{-1} for *S. aureus* (Fig. 3). As expected, when PAAs were administered alone, the irradiation of cells did not influence bacterial viability (data not shown).

In all PDT-experiments, the bacteria were incubated 10 min in the dark and then irradiated for 15-30-60 min. As previously reported, this incubation time is sufficient to ensure the interaction between PS and bacteria and the amount of light irradiated is non-toxic to bacteria albeit suitable to activate the photoinduced killing effect of the BODIPY [8].

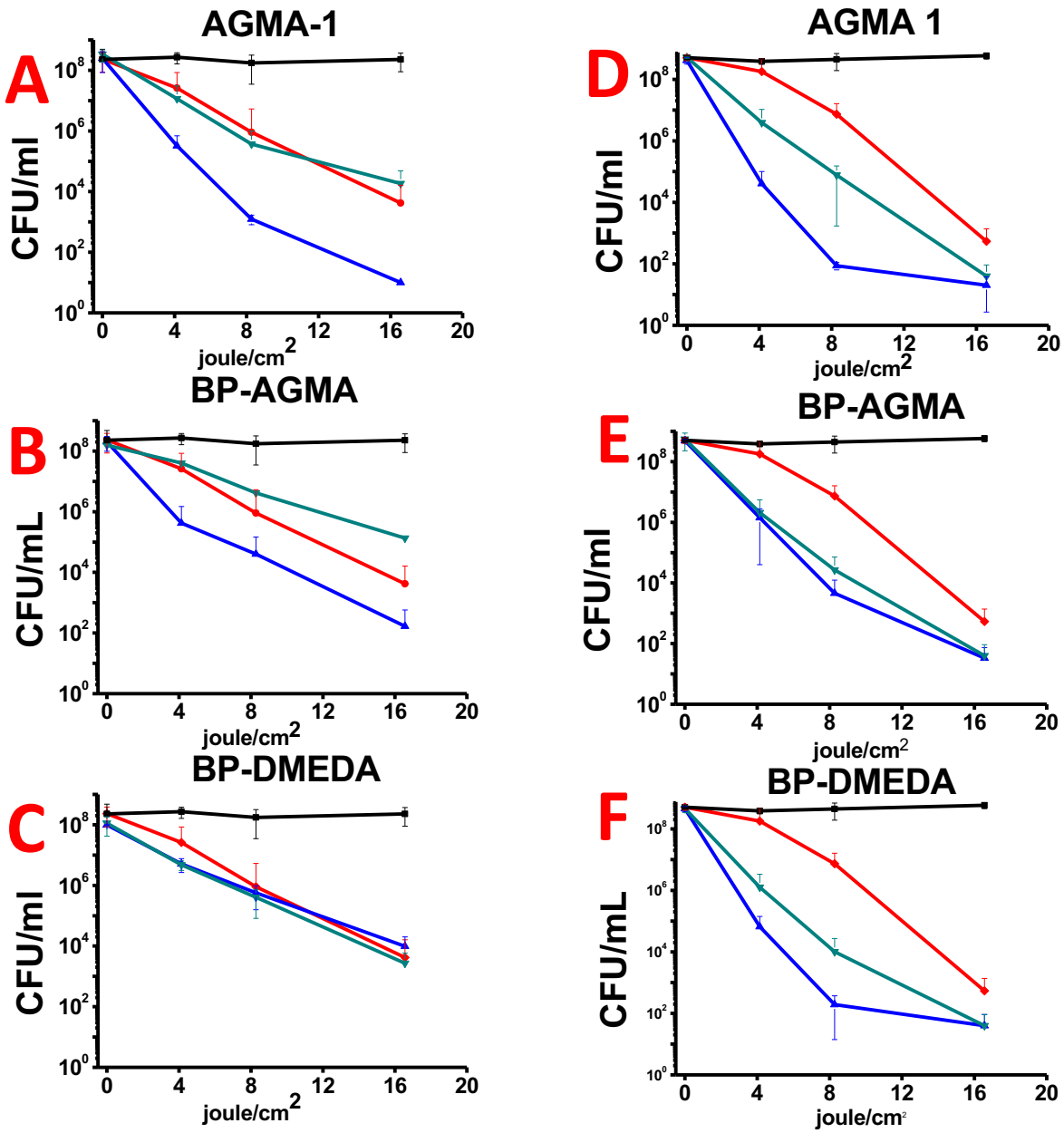


Fig. 3 Effect of PAAs on BODIPY induced photoinactivation on *E. coli* (A,B,C) and *S. aureus* (D,E,F). Bacteria were incubated in the dark for 10 min with BOD-N-Me 1 µM and 0.1 µM, respectively. Bacterial samples were added or not with PAAs 1 µg mL^{-1} and 10 µg mL^{-1}, respectively. Upon irradiation with increasing doses of light (joule cm^{-2}) cellular viability (CFU mL^{-1}) was checked. PDT experiments were performed at least three times. The black squares represent the effect of irradiation of cell culture alone (control), the red dots the effect of the PS alone under irradiation; blue triangles represent PS plus PAA (8000 Da) and overturned green triangles represent PS plus PAA (24000 Da). The PAA used in each experiment is indicated on the top of the figure.

The administered PAAs influenced the photoactivity of BOD-NMe on the two different model microorganisms depending on the PAA size and PAA type, however the killing effect was increased or equal to that obtained with photosensitizer alone (Fig. 3).

In order to avoid any toxic effect in *E. coli,* PAAs have been administered at a very low concentration, 1 μg mL^{-1}. The co-administration of AGMA-1 8000 Da and BOD-N-Me caused a 3 log units viability decrease respect with BODIPY alone at the highest energy density tested. Thus the observed increase was energy-dose dependent. On the other hand, the AGMA-1 24000 did not cause any activity enhancement. The second PAA tested (BP-AGMA) showed a lower coadjutant effect as only a decrease of 2 log units was observed as compared with PS alone, whereas the last PAA tested (BP-DMEDA) did not produce any difference in the bacterial survival with respect to the effect of the only PS.

PAAs have been administered to *S. aureus* at 10 μg mL^{-1}, a concentration higher than that used in *E. coli*. Both sizes of AGMA-1, BP-AGMA and BP-DMEDA (8000 and 24000 Da) enhanced the photokilling activity of the BODIPY alone in *S. aureus*. In particular AGMA-1 8000 and BP-DMEDA 8000 were more efficient than corresponding PAA 24000 Da at the half total irradiation time (30 min, 8.3 J cm^{-2}): AGMA1 and BP-DMEDA 8000 Da determined a decrease of survivors of about 5 log units (Fig. 3D and 3F). When the PAAs 24000 Da were administered, similar decreases were not observed. BP-AGMA 8000 and 24000 Da caused a 3 log units decrease at 30 min of irradiation (Fig. 3E).

Discussion

The PAAs tested in this work are characterized by different chemical structures consistent with the reactants used in the synthesis. Two of them (BP-DMEDA and BP-AGMA) derived from the polyaddition of dimethylethylendiamine and 4-aminobutylguanidine, respectively, to 1,4-bis acryloylpiperazine (BP). The third one derived from the polyaddition of 4-aminobutylguanidine

(agmatine) to 2,2-bisacrylamidoacetic acid (BAC). Obviously, these three PAAs have different ionic properties, therefore, they were differently charged in aqueous media. At the pH of the experiments (pH = 7.2), the number of charges for 8000 Da and 24000 Da polymers respectively were: AGMA1 12 and 36; BP-AGMA 40 and 120; BP-DMEDA 30 and 90 [16]. Moreover, AGMA1 was not purely basic. It was amphoteric, but prevailingly basic. Hence, the figure 1 represents the excess positive charges.

As far as the toxicity of PAA against the two tested bacterial strains is concerned, it is interesting to observe that *E. coli* was slightly more sensitive to PAA than *S. aureus*. Actually, up to 10 μg mL^{-1} concentration, none of the tested PAAs showed intrinsic toxicity in *S. aureus*.

Although the toxicity of these PAA was quite low against *E. coli* it was statistically significant upon treatment with AGMA1 and BP-DMEDA at 24000 Da at 2.5 and 5 μg mL^{-1}, respectively. The toxicity does not seem to correlate with the number of the positive charges, however it is noticeable that a slightly higher toxicity was observed in the case of the high molecular weight AGMA1 and BP-DMEDA.

It was considered that the studies concerning the adjutant effect of the PAAs on the photoinduced cell killing by BODIPY could be carried out, in principle, under two possible conditions concerning the PAA concentration: a) by using a partially toxic concentration; b) by using a non-toxic concentration. In the first option, a low synergistic effect between PS and PAA could be masked by the killing effect of the single substances and, therefore, difficult to be observed or properly evaluated. On the other hand, the use of non-toxic PAA concentration would allow the clear detection of whichever possible enhanced photodynamic activity, therefore this latter experimental condition has been chosen.

When the PS-PAA mixtures were used against *E. coli* and *S. aureus*, the highest killing rate effect was observed for the lower molecular weight fraction of AGMA1.

As the killing effect is certainly related to the amount of PS inside or attached to the bacteria envelope, this result seems to indicate a higher availability of the photosensitizer in the bacterium

environment, probably due to an increased interaction of the PS with the cell wall and/or a higher PS permeability, elicited by the presence of PAA.

The coadjutant effect of the higher Mw polymers (24000 Da) was less pronounced in all experiments carried out with both the microorganisms, but especially with the Gram-negative one. It is difficult to give an explanation to this result at this level of the scientific investigation. Actually an opposite result was expected with the larger polymers as higher positive charges density should increase the "detergent" effect, just like partially observed when the PAAs were tested against *E. coli*. Moreover, when the photoinduced killing effect of BOD-NMe was also considered, the higher molecular weight PAAs seems to decrease the enhancement of PS activation. We believed that a possible explanation could be found by considering the interaction of the 24000 Da samples with the external bacterium wall, thus masking the bacteria outer wall with a positively charged barrier. This barrier, conversely from the bacteria external negative charges, bears cationic groups thus repelling the equally charged PS or competing for the PS binding sites.

By concluding in this work, it was for the first time demonstrated the enhancing effect of PAAs in the BODIPY photoinduced bacteria killing. The combined effect of PAA, PS and light afforded to kill both *S. aureus* and *E. coli*. This excellent result could have been obtained after only one hour irradiation with a harmless green light in the presence of a very low concentration of a cationic BODIPY, i.e. 1.0 μM and 0.1 μM for *E.coli* and *S. aureus*, respectively.

Despite of it has been already proved that BOD-NMe can be considered a powerful antibacterial photosensitizer, the present work demonstrated that, in the presence of cationic polyamidoamine, the efficacy of BOD-NMe is remarkably further enhanced. This effect was particularly striking against the Gram-negative strain *E. coli*, which is usually more tolerant to photoinactivation with respect to the Gram-positive bacteria [17].

The three PAAs used in this study have never been tested before as antibacterial cationic polymers. Two of these (AGMA-1 and BP-DMEDA) showed a certain degree of intrinsic toxicity against the Gram-negative bacterium tested (*E. coli*), however this effect is negligible at

concentrations < 5 μg mL^{-1}. Conversely, it was ineffective against Gram-positive *S. aureus* at all tested concentrations, suggesting a selective toxicity that could be exploited. Due to the presence of two components with different mechanism of action, the PDT conditions reported here open a new strategy towards the eradication of microorganisms, in localized and light accessible infections, since it could hardly elicit the selection of resistant bacterial strains.

Compliance with ethical standards

Ethical approval The authors declare that they do not need the ethical approval from any committee.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All subjects willing to participate in this study signed an informed consent form.

Role of funding source This research was funded by the University of Insubria (Fondo di Ateneo per la Ricerca).

REFERENCES

1. Hamblin MR (2016) Antimicrobial photodynamic inactivation: a bright new technique to kill resistant microbes. Curr Opinion Microbiol 33:67-73

2. Dai T, Huang YY, Hamblin MR (2009) Photodynamic therapy for localized infections-state of the art. Photodiagnosis Photodyn Ther 6:170-188

3. Maisch T (2015) Strategies to optimize photosensitizers for photodynamic inactivation of bacteria. J Photochem Photobiol B 150:2-10

4. Punia A, He E, Lee K, Banerjee P, Yang N-L (2014) Cationic amphiphilic non-hemolytic polyacrilates with superior antibacterial activity. Chem Commun 50:7071-7074

5. Chen J, Burghart A, Derecskei-Kovacs A, Burgess K (2000) 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) dyes modified for extended conjugation and restricted bond rotations. J Org Chem 65:2900-2906

6. Li L, Han J, Nguyen B, Burgess K (2008) Syntheses and spectral properties of functionalized, water-soluble BODIPY derivatives. J Org Chem 73:1963-1970

7. Yogo T, Urano Y, Ishitsuka Y, Maniwa F, Nagano T (2005) Highly efficient and photostable photosensitizers based on BODIPY chromophore. J Am Chem Soc 127:12162-12163

8. Caruso E, Banfi S, Barbieri P, Leva B, Orlandi VT (2012) Synthesis and antibacterial activity of novel cationic BODIPY photosensitizers. J Photochem Photobiol B 114:44–51

9. Orlandi VT, Rybtke M, Caruso E, Banfi S, Tolker-Nielsen T, Barbieri P (2014) Antimicrobial and anti-biofilm effect of a novel BODIPY photosensitizer against *Pseudomonas aeruginosa* PAO1. Biofouling 30:883-91

10. Ferro S, Ricchelli F, Monti D, Mancini G, Jori G (2007) Efficient photoinactivation of methicillin-resistant Staphylococcus aureus by a novel porphyrin incorporated into a poly-cationic liposome. Int J Biochem Cell Biol 39:1026–1034

11. Yin M, Li Z, Zhou L, Dong K, Ren J, Qu X (2016) A multifunctional upconverting nanoparticle incorporated polycationic hydrogel for near-infrared triggered and synergistic treatment of drug-resistant bacteria. Nanotechnology 27:125601-7

12. Deng X, Liang Y, Peng X, Su T, Luo S, Cao J, Gu Z, He B (2015) A facile strategy to generate polymeric nanoparticles for synergistic chemo-photodynamic therapy. Chem Commun 51:4271-4274

13. de Freitas LM, Calixto GM, Chorilli M, Giusti JS, Bagnato VS, Soukos NS, Amiji MM, Fontana CR (2016) Polymeric Nanoparticle-Based Photodynamic Therapy for Chronic Periodontitis in Vivo. Int J Mol Sci 17:769-782

14. Ferruti P (2013) Poly(amidoamine)s: Past, present, and perspective. J Polym Sci Part A: Polymer Chem 51:2319–2353

15. Sasaki I, Bertani G (1965) Growth abnormalities in Hfr Derivatives of *Escherichia coli* Strain *c.* J gen Microbiol 40:365-376

16. Ranucci E, Ferruti P, Lattanzio E, Manfredi A, Rossi M, Mussini PR, Chiellini F, Bartoli C (2009) Acid-base properties of poly(amidoamine)s. J Polymer Sci Part A: Polymer Chem 47:6977–6991

17. Sperandio FF, Huang YY, Hamblin MR (2013) Antimicrobial photodynamic therapy to kill Gram-negative bacteria. Recent Pat Antiinfect Drug Discov 8:108-20