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Uptake pathways of anionic and cationic photosensitizers into bacteria

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Abstract

The effect of divalent cations (calcium and magnesium) and a permeabilizing agent (EDTA) on the uptake of a cationic photosensitizer (PS), methylene blue (MB), and two anionic PSs, rose bengal (RB) and indocyanine green (ICG), by Gram-positive *Enterococcus faecalis* and Gram-negative *Actinobacillus actinomycetemcomitans* was examined. The possible roles of multidrug efflux pumps and protein transporters in photosensitizer uptake were assessed in *E. faecalis* cells by studies using an efflux pump inhibitor (verapamil) and trypsin treatment respectively. Divalent cations enhanced the uptake and photodynamic inactivation potential of both RB and ICG in *E. faecalis* and *A. actinomycetemcomitans*, while they decreased the uptake and bacterial killing by MB. Verapamil increased the uptake of RB (possibly due to efflux pump inhibition), whereas trypsin treatment resulted in significant decrease in RB and ICG uptake. The results suggested that the uptake of anionic PSs by bacterial cells may be mediated through a combination of electrostatic charge interaction and by protein transporters, while the uptake of cationic PSs, as previously reported, is mediated by electrostatic interactions and self promoted uptake pathways.

1. Introduction

The possibility of using antimicrobial photodynamic therapy (PDT) to treat bacterial infections in locations such as the oral cavity has been shown by many *in vitro* and *in vivo* studies.[1,2] The highly reactive singlet oxygen ($^1O_2$) generated during the excitation of a photosensitizer (PS) is thought to be the principal antimicrobial agent in PDT.[3–5] The localized action of $^1O_2$ generated during PDT, implies that the PS is more effective if it is taken up into its target cell before light is delivered. Subsequently, the $^1O_2$ is better able to oxidize important cellular targets such as membrane, enzymes, and lipids that lead to bacterial killing.[2] However, membrane barriers of the bacterial cell limit the simple diffusion of PS into the bacterial cytosol. The membrane barriers of Gram-positive bacteria consist of a relatively thicker but porous cell wall made up of inter-connected peptidoglycan layers surrounding a cytoplasmic membrane.[6] The teichoic acid residues of the cell wall contribute to the negative charge and consequent

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binding sites for cationic molecules.[6] On the other hand, the cell envelope of Gram-negative bacteria is composed of an outer membrane, a thinner peptidoglycan layer and a cytoplasmic membrane. Movement of molecules across the Gram-negative cell wall is strictly regulated at the outer membrane which is rich in lipopolysaccharides (LPS).[7,8] Negatively charged LPS molecules have a strong affinity for cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), the binding of which is required for the thermodynamic stability of the outer membrane.[9]

Unlike Gram-positive bacteria, Gram-negative bacteria are less susceptible to PDT due to the membrane barrier that prevents uptake of anionic and neutral PS.[9–11] However, the initial difficulty in photodynamic inactivation (PDI) of Gram-negative bacteria was overcome either by using positively charged (cationic) PS, or by coupling or combining the PS with positively charged entities such as poly-L-lysine,[12] polyethyleneimine,[13] and polymyxin B nonapeptide (PMBN)[14] *etc.* These cationic entities were shown to enter the bacterial cytosol *via* the 'self promoted uptake pathway'.[10,15] In addition to self promoted pathway, 'protein transport machineries' present in the bacterial cell envelope could also mediate PS uptake.[16] The 'porin' class of protein transporters facilitate the uptake of low molecular weight (600–700 Da) hydrophilic compounds.[8] Although the presence of porin proteins was initially reported in the outer membrane of Gram-negative bacteria, ion selective porin proteins have also been identified on Gram-positive bacterial cell walls.[16,17] Taken together, it is understood that the functioning of self-promoted uptake pathways and protein transporters are modulated by charged entities such as cations. Therefore, the success of PDT in eliminating bacteria from anatomical sites such as root canals and periodontal pockets in the oral cavity could be influenced by the cation-rich microenvironment persisting at these sites. Although the possibility of using PDT in diseases such as root canal infection and periodontitis has been proposed by many researchers, the influence of divalent cations and the mechanism of PS uptake by oral bacteria have not been investigated thoroughly.

The goal of this study was to examine the influence of extracellular divalent cations in the uptake of the cationic PS, methylene blue (MB) and two different anionic PS, rose bengal (RB) and indocyanine green (ICG) by oral bacteria *Enterococcus faecalis* and *Actinobacillus actinomycetemcomitans*. The influence of EDTA that disrupts the outer membrane of Gram-negative bacteria by removing the divalent cations was also studied. In order to verify the possible role of intracellular calcium and protein transporters, the PS uptake was studied in *E. faecalis* cells subjected to verapamil and trypsin treatment respectively. Since divalent cations may influence the PS uptake, PDI of *E. faecalis* was also studied in the presence of $CaCl_2$. MB, RB and ICG were selected as PSs for this study as they are generally regarded as safe for medical applications, due to the lack of significant dark toxicity.[18–22]

2. Materials and methods

Bacterial culture conditions

E. faecalis (ATCC 29212), a Gram-positive bacterium, and *A. actinomycetemcomitans* (ATCC 33384), a Gram-negative bacterium, were used in this study. *E. faecalis* was grown in All Culture (AC) media (Sigma Aldrich, USA), while *A. actinomycetemcomitans* was grown in Brain Heart Infusion (BHI) broth (Difco, BD Diagnostic Systems, MD) supplemented with hemin and vitamin K. Bacterial cells in the stationary phase (over-night growth) were collected by centrifugation (3000 *g* for 10 min) and were washed with deionized (DI) water. The OD of cells suspended in deionized water was adjusted to 1 at 600 nm ($\approx 0.5–0.8 \times 10^9$ CFU mL^{-1}). Cells from 1 mL of the above suspension were collected by centrifugation and used for further studies.

Photosensitizers

MB ($C_{16}H_{18}ClN_3S{\cdot}3H_2O$, mol wt = 374), RB ($C_{20}H_2Cl_4I_4O_5Na_2$, mol wt = 1017) and ICG ($C_{43}H_{47}N_2O_6S_2Na$, mol wt = 775), with chemical structures shown in Fig. 1, were obtained from Sigma-Aldrich Chemical Co (St Louis, MO) and were used without further purification. 1 mM stock solutions of PSs in deionized water were freshly prepared each time.

Uptake and PDI of *E. faecalis* in the presence of $CaCl_2$

E. faecalis cells (10^9 CFU mL^{-1}) were incubated with increasing concentrations of MB, RB and ICG (0, 6.25, 12.5, 25, 50, and 100 μM) in the presence of 50 mM $CaCl_2$ for 30 min in dark. After the incubation, washed bacterial cells were treated with 1 mL of 2% SDS for 14 h at room temperature in order to extract the cell-bound PS. The supernatant solution after centrifugation was taken for PS quantification. Quantification of PS was done spectrophotometrically at the respective absorption maxima of the PS used (MB: 664 nm, RB: 532 nm and ICG: 800 nm). Calibration curves were constructed for each PS in 2% SDS.

For photoinactivation studies, 100 μL aliquots of the cell suspensions were transferred to 96 well polystyrene plates (Sterilin, Barloworld Scientific Ltd, Staffordshire, UK). These bacterial suspensions were irradiated with the corresponding wavelength of light. A diode laser of wavelength 664 nm was used for cells treated with MB (fluence 1.8 J cm^{-2}, fluence rate of 0.09 W cm^{-2}) while a diode laser with wavelength at 800 nm was used to irradiate the cells treated with ICG (fluence 3.6 J cm^{-2}, fluence rate of 0.30156 W cm^{-2}) (Power Technology Inc, Little Rock, AR, USA). A non-coherent light source with a band-pass filter (540 ± 15 nm) (Lumacare, USA) was used for irradiating the RB-treated cells at a fluence of 1.8 J cm^{-2} at a fluence rate of 0.229 W cm^{-2}. The cell survival was determined after 10-fold serial dilution of the cell suspension and plating onto BHI agar plates. The number of colony forming units was determined after incubating the plates at 37 °C for 24 h. The $\log_{10}$ number of surviving bacterial cells after the photoinactivation was plotted against the concentration of the PS.

Chemical treatments and photosensitizer uptake assay

The cells obtained after washing and adjusting the optical density were divided into three different groups. The first group of cells was subjected to increasing concentrations of $CaCl_2$ (0, 6.25, 12.5, 25 and 50 mM) in water. Similarly, the second group of cells was subjected to increasing concentrations of $MgCl_2$ (0, 6.25, 12.5, 25 and 50 mM) in water and the third group was treated with increasing concentrations of EDTA (pH 8) (0, 6.25, 12.5, 25 and 50 mM). The cells were exposed to respective chemical solutions at 37 °C for 30 min and the treatment was quenched by pelleting the cells by centrifugation and washing with deionized water to remove the unbound chemicals.

One mL of freshly prepared 50 μM MB, RB, or ICG in deionized water was added to the pelleted cells from the above three groups ($CaCl_2$, $MgCl_2$ and EDTA) and were incubated for 30 min at 37 °C. The extent of PS binding to the bacterial cells was estimated spectrophotometrically as described in the preceding experiment.

Effect of trypsinization and verapamil on photosensitizer uptake

E. faecalis cell pellets (10^9 CFU) were mixed with 1 mL of trypsin at 0.1% and 0.5% solution, equivalent to 18 or 90 TAME μM units, (Gibco, Invitrogen Corporation) for 30 min at 37 °C. The treatment was terminated by harvesting the cells and washing them in deionized water. The collected cells were suspended in 50 μM of MB, RB or ICG for 30 min at 37 °C and the PS uptake was assayed as described. *E. faecalis* cells were treated with calcium channel blocker, verapamil (Sigma-Aldrich, MO, USA) at a concentration of 10 μM and 100 μM for 30 min at 37 °C. The treatment was stopped by harvesting the cells and washing them in

deionized water. The collected cells were suspended in 50 μM of MB, RB or ICG for 30 min at 37 °C and were assayed for PS uptake as described earlier.

Statistics

All experiments were repeated in triplicate with three samples per group (total $n = 9$). Two-way ANOVA was conducted to study statistical significance. Significance levels were set at $P < 0.05$.

3. Results

Effect of divalent cations and EDTA treatment on MB, RB and ICG uptake and PDI of Gram-positive *E. faecalis*

Treatment with divalent cations ($CaCl_2$ and $MgCl_2$) and EDTA was studied in two different experiments. Firstly, the concentration of $CaCl_2$ was fixed at 0 to 50 mM and the concentration of PS varied between 0 and 100 μM (Fig. 2A–C); in these experiments the PDI of bacteria was also studied after irradiating with 1.8 or 3.6 J cm^{-2} of the appropriate wavelength light (Fig. 3A–C). Secondly, the concentration of PS was kept constant and the concentrations of divalent cations or EDTA was varied between 0 and 50 mM (Fig. 4A–C). Divalent cations had completely opposite effects on the uptake of PS by *E. faecalis* depending on the chemical nature of the PSs. Calcium chloride reduced the uptake of cationic PS MB into *E. faecalis* (Fig. 2A) by approximately 50% at all PS concentrations ($P < 0.001$). In contrast there was a large increase (up to ten fold, $P < 0.0001$) in uptake of anionic PS RB (Fig. 2B) in the presence of calcium chloride. Likewise there was a smaller increase ($P < 0.05$) in the uptake of anionic PS ICG in the presence of calcium ions but only at higher ICG concentrations (Fig. 2C).

These differences in PSs uptake between bacteria incubated in the absence and presence of calcium were reflected in the extent of PDI measured after illumination by low doses of visible light. Fig. 3A shows that in the presence of calcium chloride, MB-mediated PDI of *E. faecalis* was dramatically lower when compared to PDI carried out it in the absence of calcium chloride. Complete inactivation of bacteria was achieved with 6.25 μM of MB in the absence of calcium chloride, while 100 μM of MB was required to achieve complete bacterial inactivation in the presence of calcium chloride. In the case of RB the presence of calcium chloride gave the opposite effect, in that PDI was potentiated not protected. Fig. 3B shows that only a modest level of bacterial inactivation was achieved by RB with 1.8 J cm^{-2} at 540 nm light, while addition of calcium chloride caused complete inactivation of bacterial at 25 μM of RB and light. The PDI of bacteria with ICG and 800 nm light was disappointing. Only marginal PDI was observed after 3.6 J cm^{-2} was delivered, but the bacterial inactivation was still potentiated in the presence of calcium chloride (Fig. 3C).

We then investigated the effect of varying the concentration of the divalent cations (Ca^{2+} and Mg^{2+}) and divalent metal chelator EDTA on the uptake of the PSs. In Fig. 4A it can be seen that both calcium chloride and magnesium chloride inhibit the uptake of MB by *E. faecalis* in a similar fashion, with a proportionately larger drop at 5 mM Ca^{2+}/Mg^{2+} concentration and a lesser reduction at higher concentrations. EDTA treatment gave a small increase in uptake at 5 mM that disappeared at higher EDTA concentrations. Fig. 4B shows that the increase in RB uptake produced by divalent cation treatment was mainly seen with 5 mM, and lesser increase in uptake with further increase in concentration of Ca^{2+}/Mg^{2+}. Here, calcium appeared to have a more pronounced effect in increasing RB uptake than did magnesium. EDTA had very little effect on the uptake of RB by *E. faecalis.* However the increase in uptake of ICG by *E. faecalis* after treatment with Ca^{2+}/Mg^{2+} was more pronounced (Fig. 4C). Treatment with 5 mM Ca^{2+} or Mg^{2+} increased the uptake ten-fold, with very little further increase after

increasing the cation concentration up to 50 mM. There was a significant increase (3-fold) in ICG uptake with increasing EDTA concentration up to 50 mM.

Effect of divalent cations and EDTA treatment on MB and ICG uptake by Gram-negative *A. actinomycetemcomitans*

When *A. actinomycetemcomitans* was treated with increasing concentrations of calcium chloride and magnesium chloride there was a dramatic decrease in the uptake of MB; even 5 mM Ca^{2+}/Mg^{2+} reduced the uptake by more than 90% (Fig. 5A). There was a less pronounced decrease in uptake of MB when the cells were treated with increasing EDTA concentrations. The uptake was about 60% of the control value when the concentration of EDTA reached 50 mM.

Again opposite effects were seen with the anionic PS, ICG. Fig. 5B shows a dramatic increase in uptake of ICG by *A. actinomycetemcomitans* in the presence of calcium chloride or magnesium chloride (to a slightly lesser extent). The majority of the increase was seen at 5 mM Ca^{2+}/Mg^{2+} when the uptake was almost 20-fold higher. Increasing concentrations of EDTA gave a small increase (doubling) in uptake of ICG, by *A. actinomycetemcomitans* (Fig. 5B).

Effect of trypsin and verapamil on MB, RB and ICG uptake by *E. faecalis*

The effect of two different concentrations of trypsin and of verapamil on photosensitizer uptake by *E. faecalis* cells are shown in Fig. 6. Treatment with verapamil increased the uptake of MB (8 and 12% increase for 10 and 100 μM verapamil respectively) and had a significant effect on the uptake of ICG only at 100 μM of verapamil (10% increase). However, verapamil treatment had a large and significant effect on the uptake of RB (increase of 63% with 10 μM and of 136% with 100 μM). Trypsin treatment of cells had no significant effect on the uptake of MB by *E. faecalis* cells, whereas the uptake of ICG was greatly reduced after trypsinization (67% reduction with 0.1% and 74% reduction with 0.5%). The uptake of RB was also reduced after trypsin treatment but to a lesser extent than ICG (20% with 0.1% and 40% with 0.5%).

4. Discussion

An understanding of the mechanisms and factors influencing PS uptake by bacteria is necessary for optimizing antibacterial PDT regimens. This report provides evidence for the first time that anionic PS such as RB and ICG are not taken up into bacterial cells *via* simple diffusion. We studied the role of divalent cations in the uptake of PS and photoinactivation of bacteria for two reasons: (i) the functioning of transport machineries in bacteria are regulated by ‘ions’ and (ii) often anatomical sites associated with bacterial biofilm infections and bacterial envelope are rich in cations such as Ca^{2+} and Mg^{2+}. The results from this study clearly demonstrated that divalent cations increase the uptake of anionic PSs (ICG and RB) by both Gram-negative and Gram-positive bacterial cells. Furthermore the uptake of the anionic PS is sensitive to trypsin suggesting that uptake is mediated by a protein transporter.

MB is a positively-charged, hydrophilic phenothiazinium PS with an absorbance maximum of 664 nm[23] (Fig. 1). The capability of MB to generate singlet oxygen and other reactive oxygen species when bound to negatively-charged interfaces has been reported by earlier workers.[24,25] *In vitro* and *in vivo* studies have demonstrated the antimicrobial activity of PDT using MB, implying the possibility of using MB for eradicating bacterial infections in humans.[26] There are many studies showing that the mechanism of uptake of cationic PS such as MB by Gram-negative bacteria is by the so-called “self-promoted uptake” pathway.[15,26] This pathway involves the binding of the cationic molecules to LPS that results in the progressive displacement of divalent cations thereby weakening the outer membrane. The initial binding

of the positively charged PS to the outer LPS layer of Gram-negative cells displaces the divalent cations (Ca^{2+} and Mg^{2+}) that normally stabilize the LPS structure *via* electrostatic bonds. The destabilization of the LPS coat results in the formation of "cracks" in the permeability barrier. The presence of cationic dye in the medium results in the progressive widening of the crack in the LPS layer. Conversely, the efficiency of the self-promoted uptake pathway is reduced by the presence of divalent cations that stabilize the LPS layer.[8,27] The surface adsorption of divalent cations in $CaCl_2$ and $MgCl_2$ treated cells could have competitively reduced the membrane disruption by MB molecules, reducing its uptake by *A. actinomycetemcomitans.* Similarly, the competitive binding of divalent cations to the negative entities on the Gram-positive cell wall of *E. faecalis* is thought to reduce the electrostatic binding sites for MB molecules (see below). Correspondingly, there was reduction in the PDI (using MB) of both these bacterial species when the cells were subjected to divalent cation treatment. However, as evident from this study, an increase in the MB uptake and photoinactivation of bacteria in the presence of divalent cations could be achieved by increasing the molar concentration of MB in the sensitizing solution. These observations point toward competitive binding of MB to LPS and cell wall of Gram-negative and Gram-positive bacteria, respectively. The data from our studies correlate with observations made by Usacheva *et al.* on the interaction of Ca^{2+} with bactericidal action of phenothiazine dyes.[27] Similarly, Lambrechts *et al.* showed that the uptake and subsequent phototoxicity of a cationic porphyrin by both Gram-negative *Pseudomonas aeruginosa* and by Gram-positive *Streptococcus aureus*, was reduced by concentrations of calcium chloride up to 5 mM.[28] The reduction in killing was more pronounced in the case of *P. aeruginosa* (a Gram-negative bacterium) because of the above mentioned self-promoted uptake pathway.

The major effect of Ca^{2+} and Mg^{2+} treatment in increasing the uptake of the anionic PSs, RB and ICG by both Gram-positive *E. faecalis* and Gram-negative *A. actinomycetemcomitans* was unexpected. The two major calcium-binding sites on Gram-positive cell surfaces are carboxylate groups (in proteins and peptidoglycan cross-links) and phosphate groups (in lipoteichoic and teichoic acid).[29,30] One possible explanation for our observation, therefore, is that the added divalent cations bind to these carboxylate and phosphate groups and neutralize the anionic charges, thus reducing the repulsion of anionic PS by these negative charges on the cell wall. In addition, divalent cations may also influence the functioning of transport proteins, *e.g.* porins (see below).

Earlier studies from our laboratory have indicated that the surface layer of *E. faecalis* (Gram-positive bacteria) can be partially removed by EDTA treatment.[31] EDTA is reported to cause cell wall damage in bacteria by chelating the divalent cations (Mg^{2+} and Ca^{2+}).[7] The removal of divalent cations that bind together the negatively-charged cell wall molecules, make bacterial cell membrane permeable to substances. The membrane-disrupting action of EDTA has been applied to enhance the effect of antibacterial agents such as antibiotics.[32,33] EDTA has been shown to enhance PDI of Gram-negative bacteria by facilitating the penetration of cationic and anionic dyes through the outer membrane.[11] However, as observed in our studies, the treatment of bacterial cells with EDTA had only a minor effect (generally an overall increase) on the uptake of both cationic (MB) and anionic (RB and ICG) PS. This overall increase may be explained by increased PS diffusion through the disrupted outer membrane. In Gram-negative *A. actinomycetemcomitans*, however, there was actually a reduction in MB uptake on increasing the EDTA concentration. The removal of negatively charged molecules (LPS, lipoteichoic acid *etc.*) from the cell wall of bacteria by previous EDTA treatment could have resulted in the reduction of MB binding sites. The observation suggests that the cell wall of bacteria does not restrict MB uptake but provides binding sites for MB. Furthermore, the uptake of MB by bacteria was not affected by trypsin treatment, suggesting the absence of any protein transporter involved in its uptake. Taken together, the data suggested the association of MB with surface layer of bacteria. This observation is in agreement with early studies by

Bhatti *et al.* who showed the binding preference of Toluidine Blue O (a phenothiazine dye) to the outer membrane components of bacteria.[34]

Initially, we used verapamil as a calcium-channel blocker. The reasoning was that treating the cells with verapamil would affect the intracellular concentration of Ca^{2+}. However, subjecting *E. faecalis* cells to 10 or 100 μM verapamil gave only minor increases (about 10%) in uptake of MB and ICG. However major increases were observed in the uptake of RB by *E. faecalis* in the presence of 10 μM or 100 μM verapamil. Verapamil may have another relevant function in this situation. We have reported that many antimicrobial PSs are substrates of microbial multi-drug efflux pumps (MEP).[35] Treating bacteria with a small molecule inhibitor of MEP can dramatically potentiate PDI when the PS is a particularly good substrate of the MEP in question.[36] There have been recent reports that *E. faecalis* expresses a MEP of the ABC transporter class termed EfrAB, and moreover that EfrAB is inhibited by verapamil as well as reserpine, and sodium-ovanadate.[37] *E. faecalis* also expresses a MEP from a different class (major facilitator superfamily) named emeA that is a homolog of NorA from *S. aureus.*[38] This MEP is also inhibited by verapamil. It remains to be determined whether RB is a substrate of either of these *E. faecalis* efflux pumps.

Interestingly, treating *E. faecalis* cells with trypsin showed significant decrease in the uptake of both RB and ICG. Subjecting bacterial cells to sub-lethal concentrations of trypsin results in the breakdown and functional impairment of cell-wall associated proteins.[39] In this regard, the decrease in anionic PS uptake after trypsinization of *E. faecalis* cells indicated the role of protein transport machinery in the uptake of these molecules. Porin proteins are a class of transport proteins that mediate the transport of substances across the outer membrane of Gram-negative bacteria.[8] The presence of an anion specific porin channel in Gram-positive bacterium *Corynebacterium glutamicum* was reported by Costa Riu *et al.* in 2003.[17] Further studies conducted on the structure of porin have implied the possible role of divalent cations in their functional regulation, a possible reason for the increased anionic PSs uptake in the presence of divalent cations observed in our study.[40] When the above observations and the results from this investigation are combined the possible role of ‘porin proteins’ in the uptake of anionic PS cannot be excluded.

The relative ineffectiveness of ICG as an antimicrobial PS was not surprising when compared to MB and RB. ICG has been reported to have only a low singlet oxygen quantum yield,[41] and to be able to mediate PDI of cancer cells at relatively high concentrations and fluences.[42] It has been clinically tested as a topically applied PS together with NIR light for acne therapy.[43]

In conclusion, these studies suggest that divalent cations play an important role in PS uptake by bacterial cells. In situations where divalent cations are not likely to be present in high concentrations, then cationic PS such as MB could be used. Previous studies from our group have shown that bacteria growing as a biofilm on mineralized tissues such as dentine can accumulate minerals.[44,45] The high mineral content may limit the applicability of MB as a photosensitizer in treating dental infections, where PDI is likely to be a choice of treatment.[46] In these situations an anionic PS such as RB may be a good choice. However it should be noted that RB is only highly active against Gram-positive species. It may be possible to combine a cationic PS with an anionic PS for specific applications. Potentiation of antimicrobial PDT by inhibitors of microbial efflux pumps is a newly emerging field and worthy of more study.

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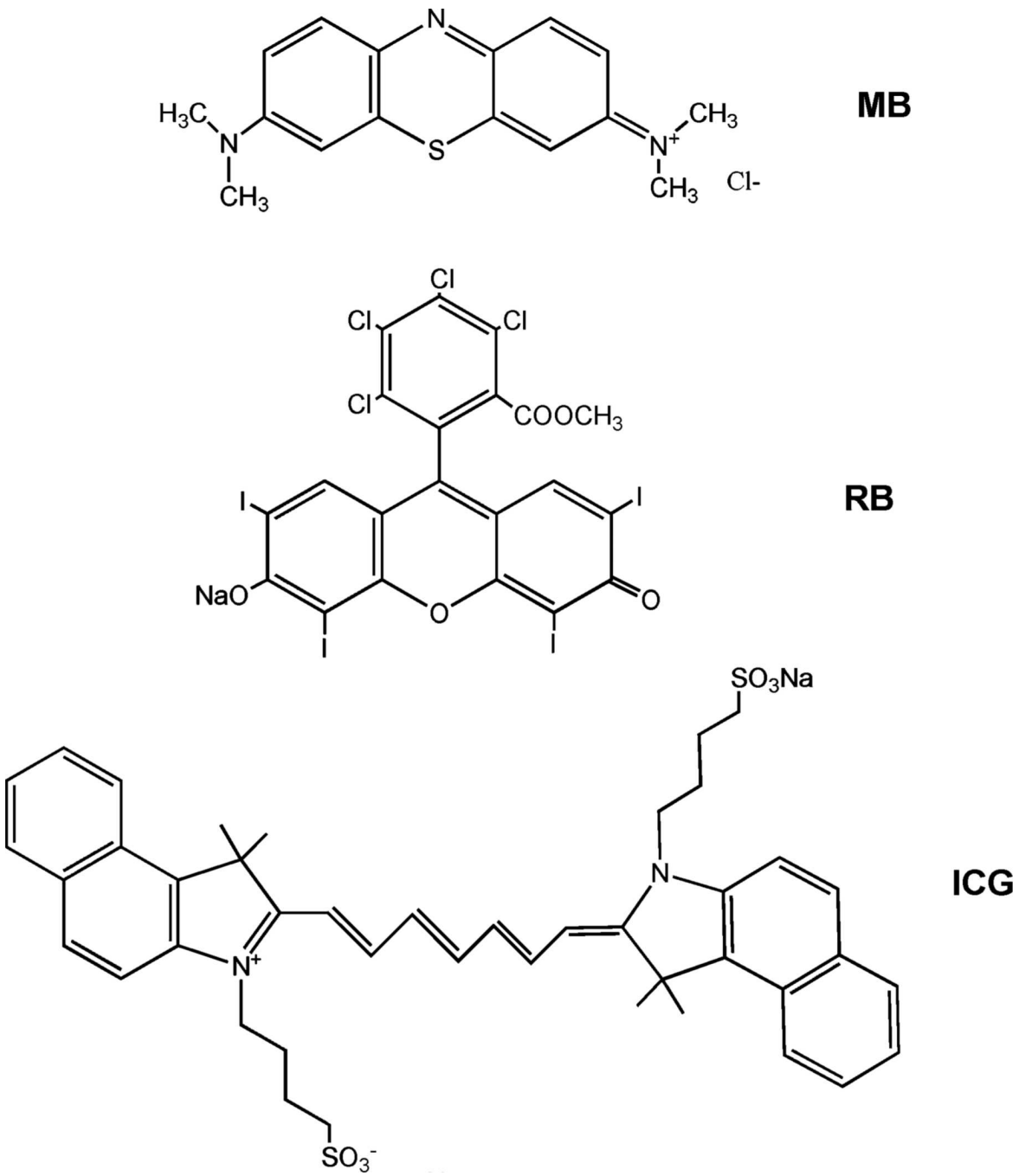

Fig. 1.
Chemical structures of the PSs used.

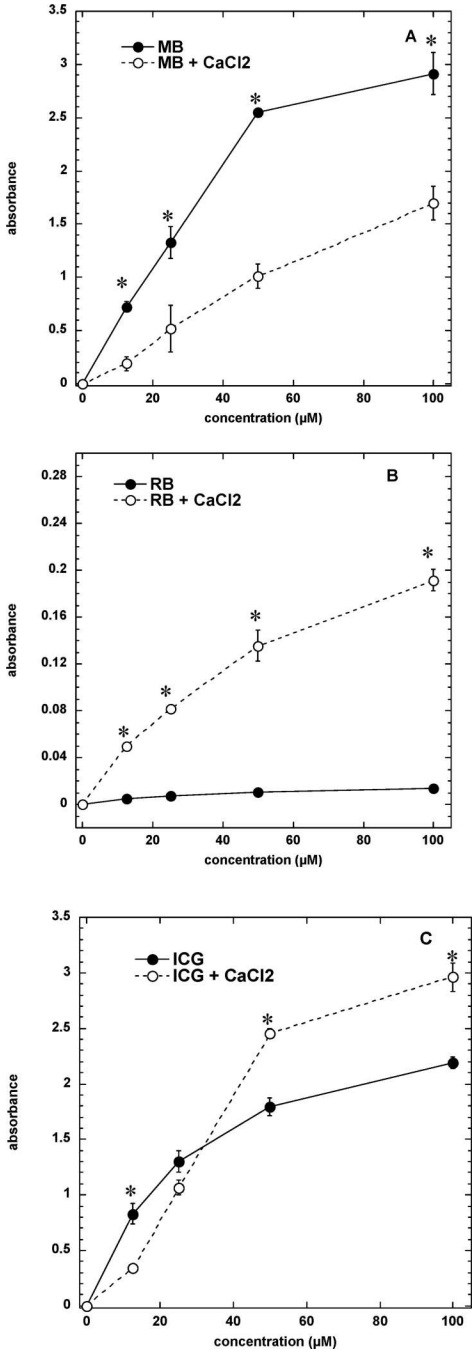

Fig. 2.

Uptake of increasing concentrations of PS (A, MB; B, RB and C, ICG) by *E. faecalis* cells incubated for 30 min with and without added $CaCl_2$ (50 mM). (* indicates the statistical significance ($p < 0.05$) between the absorbance values of $CaCl_2$ treated and untreated groups.)

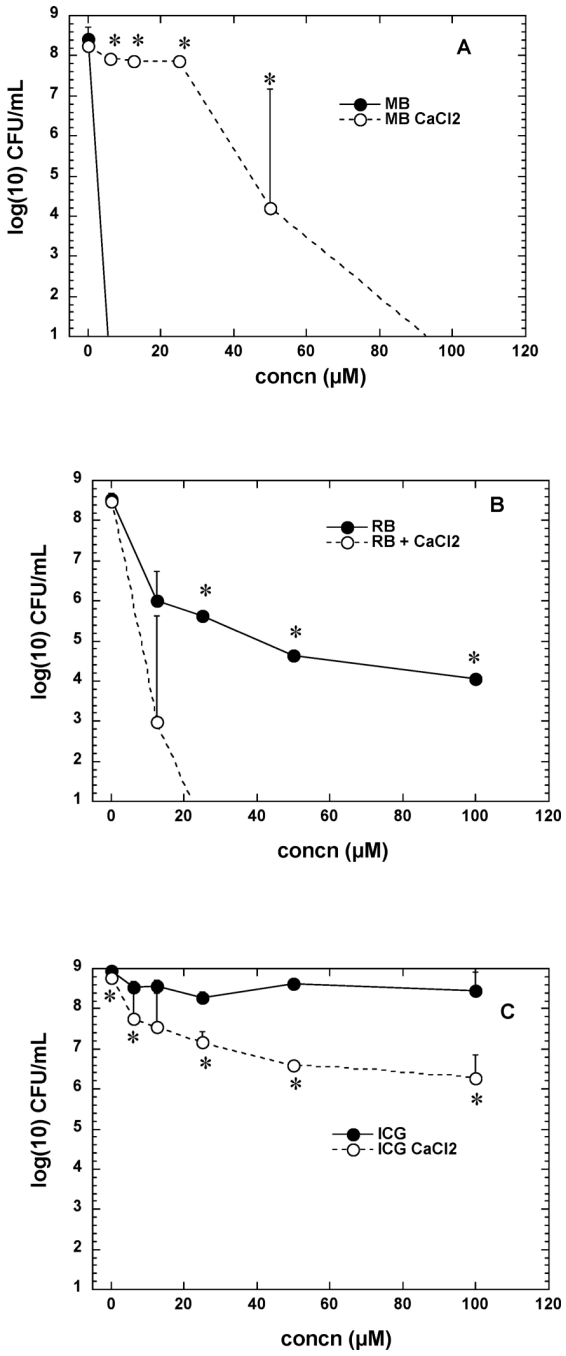

Fig. 3.

Photoinactivation of *E. faecalis* cells incubated with increasing concentrations of PS with and without added $CaCl_2$ (50 mM) as described in Fig. 2. A, MB and 1.8 J cm^{-2} of 664 nm light; B, RB and 1.8 J cm^{-2} of 540 ± 8 nm light; C, ICG and 3.6 J cm^{-2} of 810 nm light. Survival fractions are calculated with respect to untreated control cells. (* indicates the statistical significance ($p < 0.05$) between the $\log_{10}$ number of surviving bacteria in $CaCl_2$ treated and untreated groups.)

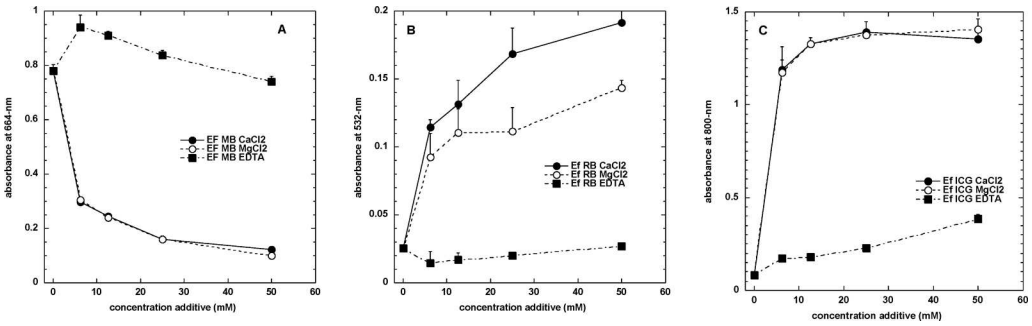

Fig. 4.

Uptake of PS (50 μM) by *E. faecalis* cells with increasing concentrations of $CaCl_2$, $MgCl_2$ or EDTA. A, MB; B, RB; C, ICG.

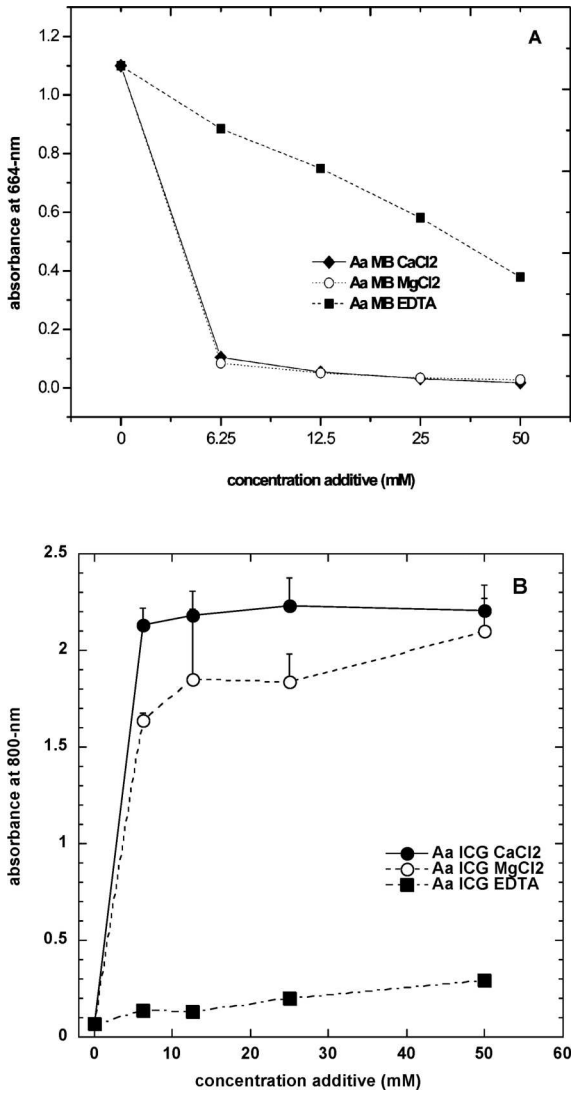

Fig. 5.

Uptake of PS (50 μM, A, MB; B, ICG) by *A. actinomycetemcomitans* cells with increasing concentrations of $CaCl_2$, $MgCl_2$ or EDTA.

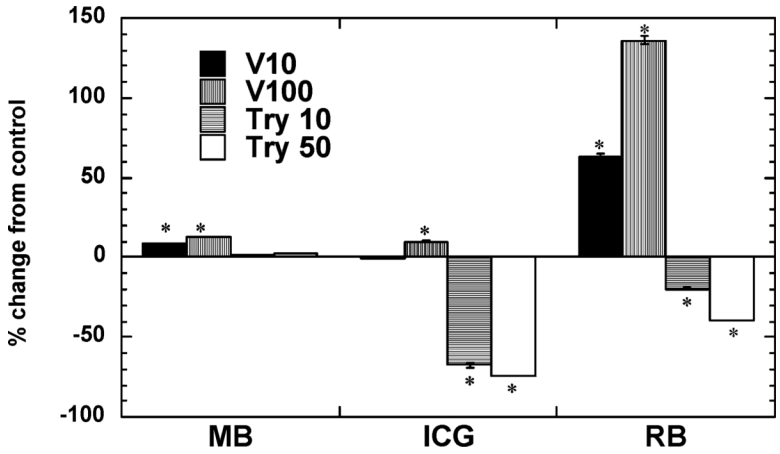

Fig. 6.

The percentage difference in uptake of PS by *E. faecalis* on treatment of the cells with verapamil (10 μM and 100 μM) and trypsin (0.1% and 0.5%) compared to control. + sign indicates an increased uptake and - sign indicate a decreased uptake in comparison to control. Trypsinisation of bacterial cells significantly reduced the uptake of ICG and RB. (* indicates the statistical significance ($p < 0.05$) in comparison to the control group receiving no treatment.)