Self-emulsifying drug delivery systems containing hydrophobic ion pairs of polymyxin B and agaric acid: A decisive strategy for enhanced antimicrobial activity

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1. Introduction

In the last century, discovery of antibiotics radically improved the treatment of microbial infection [1]. Over the decades, however, antibiotics lost efficiency due to development of resistance by pathogens limiting the treatment options for microbial infections. As there are almost no new antibiotics in the pipeline of the pharmaceutical industry, the situation is getting even worse causing global crisis [2]. Due to combination with potent auxiliary agents, however, the efficacy of many well-established antibiotics can likely be crucially improved. For instance, the addition of potentiating and sensitizing agents that synergistically act with antibiotics can enhance their efficacy [3]. One of such convincible potentiating agents is citric acid that synergistically acts with antibiotics [4]. As a chelator with three carboxylate binding sites it forms stable complexes with divalent metal ions. By binding calcium and magnesium ions citric acid destabilizes microbial membranes and increases their permeability [5]. In combination with antibiotics it can therefore enhance their penetration into microbial cells [6,7]. In order to achieve a high antimicrobial activity, however, the antibiotic should ideally reach together with citric acid the target site which is from the drug delivery point of view a great challenge. To achieve this goal, both active species have to be incorporated into a single delivery system capable of efficiently reaching the target site and providing their synchronized release there. On one hand, oily droplets of nanoemulsions seem to be promising formulations because of their lipophilic nature and small size permeating the mucus layer and microbial biofilm [8–10]. On the other hand, a promising strategy to achieve a synchronized release is the formation of complexes between cationic antibiotics with citric acid. As such complexes, however, cannot be incorporated in SEDDS because of their hydrophilic character, the lipophilic counter part of citric acid α-hexadecyl-citric acid commonly known as agaric acid (AA) might be suitable alternative. So far, however, its potential use as potentiating agent for antibiotics has not been evaluated at all.

It was therefore the aim of this study to form hydrophobic ion pairs (HIPs) between a cationic model antibiotic and agaric acid and to evaluate the potential of this complex having been incorporated in SEDDS. As model, antibiotic, polymyxin B (PMB) was chosen as this drug is first choice for treatment of a wide range of microbial infections [11]. HIPs were characterized via precipitation efficiency, ζ potential and FTIR analyses. SEDDS containing HIPs were evaluated for size distribution and binding affinity towards calcium and magnesium and antimicrobial activity using E. coli as representative bacterial strain.

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2. Material and methods

Polymyxin B sulfate was purchased from Molekula GmbH (Munich, Germany). Macrogolgycerol ricinoleate (Cremophor EL), macrogolgycerol hydroxystearate, castor oil, and glycercine monocaprylocaprate were a gift from Gattefossé (Lyon, France). Dimethyl sulfoxide (DMSO), agaric acid, magnesium chloride (MgCl₂), calcium chloride (CaCl₂), sodium hydroxide, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, tribasic sodium phosphate and acetonitrile were purchased from Sigma-Aldrich (Vienna, Austria).

2.1. Hydrophobic ion pairing (HIP)

HIPs were formed between the cationic PMB and the anionic AA. As PMB is more stable at acidic pH [12,13], it was dissolved in 0.01 M HCl and demineralized water was added to reach a final concentration of 2.6 mg/mL. Similarly, AA was neutralized with 2 M NaOH, pH was adjusted to 7.5 using 5 M HCl and demineralized water was added to reach a final concentration of 1.00 mg/mL. Therefore, the solution of PMB was added dropwise to the solution of AA in volumes corresponding to molar ratios of PMB to AA of 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6 under stirring at 300 rpm for 2 h. Precipitated HIPs were separated by centrifugation at 10000 rcf for 15 min. The collected precipitates were washed three times with demineralized water and lyophilized at −80 °C. HIPs were stored at 4 °C for further use [14].

2.2. Characterization of the HIPs formation process

The precipitation efficiency of formed HIPs was assessed by quantifying the amount of PMB remaining in the supernatant after centrifugation using UV spectroscopy [15]. The amount of remaining PMB was determined using a calibration curve in a concentration range of 3-75 μg/mL of PMB at wavelength of 210 nm. Precipitation efficiency was calculated in percentage by using the following equation:

\[
\text{Precipitation efficiency} \, [%] = 100 \left( \frac{\text{PMB conc after HIP}}{\text{PMB conc before HIP}} \right) \left( \frac{100}{1} \right) \tag{1}
\]

For evaluation of \( \zeta \) potential changes due to HIPs formation, 0.5 mL of 0.1 % (m/v) PMB solution was prepared in 0.01 M HCl and AA solutions were prepared in 0.5 mL of 0.01 M NaOH in concentrations corresponding to molar ratios of PMB to AA of 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6 in the following step. Each solution of AA was added dropwise to PMB solution under constant stirring at 400 rpm. Thereafter, the potential of the resulting precipitates was measured utilizing Zeta sizer Nano-ZSP (Malvern Instruments, Worcestershire, UK) [16].

In order to determine the intermolecular interactions of HIPs, ATR-FTIR spectra of HIPs, PMB and AA were recorded on FT/IR spectrophotometer (Bruker Alpha FT-IR Platinum) between 4000 and 400 cm\(^{-1}\) in transmittance mode.

2.3. Determination of partition coefficient (log D)

In order to assess the lipophilicity impart of PMB HIPs, n-butanol/water partition coefficient was determined according to the method previously described by our research group. Briefly, 1 mg of the PMB HIPs or 1 mg of PMB was added to 1 mL of n-butanol/water (1:1) and vortexed at 1000 rpm for 24 h. Afterwards, samples were centrifuged at 10,000 rcf for 10 min and 50 μL aliquots were withdrawn from each phase. Aliquots were prepared by taking 100 μL from n-butanol and water phase by diluting with 900 μL of methanol and water, respectively. Each sample was analyzed using 0.1 M tribasic sodium phosphate and acetonitrile (77:23) as mobile phase at a flow rate of 1 mL/min via HPLC fitted with C18 column (4.6-mm x 25-cm) at 210 nm. n-Butanol/water partition coefficient was determined by calculating ratio of concentration of PMB in n-butanol and water phase as function of logarithm.

\[
\log D = \log_{10} \left( \frac{\text{concentration of PMB in n-butanol phase}}{\text{concentration of PMB in water phase}} \right) \tag{2}
\]

Distribution coefficient (log D) of PMB HIPs between SEDDS pre-concentrate and 0.1 M PBS pH 7.4 as release medium was determined by quantifying the maximum solubility of PMB HIPs in SEDDS pre-concentrate and in the release medium [17]. Log D was calculated by using following equation:

\[
\log D = \log \frac{S_{\text{SEDDS}}}{SRM} \tag{3}
\]

where

\[
S_{\text{SEDDS}} = \text{maximum solubility in SEDDS pre-concentrate}
\]

\[
SRM = \text{maximum solubility in the release medium}
\]

2.4. Dissociation studies

Dissociation studies were performed with the PMB HIPs in 50 mM and 100 mM NaCl solutions as well as in 50 mM and 100 mM phosphate buffer pH 5.5 and pH 7.5. For this purpose, 1 mg of PMB HIPs was dispersed in 1 mL of 50 mM and 100 mM NaCl solution and in 50 mM and 100 mM phosphate buffer of pH 5.5 and pH 7.5. Dispersed HIPs were stirred at 400 rpm at 37 °C and absorption was measured at 210 nm after 4 and 8 h [14].

2.5. Preparation and characterization of SEDDS

For SEDDS preparation, castor oil, macrogolgycerol ricinoleate, macrogolgycerol hydroxyxystearate, glycercine monocaprylocaprate and dimethyl sulfoxide (DMSO) were used as excipients. In order to prepare AA loaded SEDDS, AA was dissolved in a mixture of macrogolgycerol ricinoleate and macrogolgycerol hydroxyxystearate (1:1) via ultrasonication and vortexing. PMB HIPs loaded SEDDS were prepared by dissolving HIPs in a mixture of glycercine monocaprylocaprate/DMSO (1:1) via vortexing. The components were mixed in ratios as listed in Table 1 via mixing at 1200 rpm at 37 °C using a theromixer (Thermomixer comfort, Eppendorf, Germany). These SEDDS pre-concentrates were diluted in 50 mM phosphate buffer pH 7.4 by vortexing. Subsequently, formulations were centrifuged for 5 min at 12100 rcf and visually examined regarding stability of formed emulsions. Furthermore, mean droplet size, polydispersity index and \( \zeta \) potential were determined by dynamic light scattering using a Zeta sizer Nano ZSP (Malvern Instruments, Worcestershire, UK).

2.6. Cytotoxicity assay

Cell viability assay was performed on all SEDDS formulations of different compositions as explained in Table 1 in concentrations of 0.1, 0.2, 0.5 and 1% (v/v) by resazurin reduction assay on Caco-2 cells. Culture of cells was prepared in 96-well plates at a concentration of 3.5 × 10⁴ cells per well. To culture cells, Minimum Essential Medium (MEM) was used as growth medium at 37 °C maintaining 95% humidity and 5% CO₂ atmosphere for 14 days. MEM was employed as a negative control while Triton X100 (1% w/v) as a positive control. SEDDS in a concentration of 0.1, 0.2, 0.5 and 1% were dispersed in 500 μL of MEM without phenol red and added to the cell layer in order to incubate for 4 h. Afterwards, cells were washed with 500 μL of 10 mM phosphate buffered saline pH 7.4 and incubated with 500 μL of resazurin solution for 3 h. 100 μL of supernatant was taken from each well in duplicate and fluorescence was analyzed using an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell viability was calculated as percentage
of viable cells considering viability of cells 100% treated with MEM (negative control) using following equation [18]:

\[
\text{Viable cell} \% = \frac{\text{Experimental values} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100
\]  

(3)

2.7. Calcium and magnesium binding studies

2.7.1. Binding capacity studies with citric acid and agaric acid

In this study calcium and magnesium binding capacity of citric acid and agaric acid was determined by complexometric titration according to a method previously described by our research group with some modification [19]. For this purpose, ammonia buffer was prepared by dissolving 3.50 mg of ammonium chloride in 28.5 mL of ammonia solution (35.6%) and diluted with demineralized water to a final volume of 50 mL. Demineralized water was used as solvent for titration by adjusting pH to 8.5 using 0.01 M NaOH. AA was neutralized with 2 M HCl and demineralized water was added to reach a final concentration of 1.00 mg/mL. Similarly, citric acid was dissolved in 0.01 M NaOH, pH was adjusted to 8.5 using 0.1 M HCl and demineralized water was added to reach a final concentration of 1.00 mg/mL. Eriochrome Black T (EBT) solution was prepared by dissolving 20 mg of EBT in 500 mL of ethanol and added to 1.5 mL of ammonia buffer. Briefly, 5 mL of EBT solution was added to citric acid and agaric acid solutions and titrated with 10.0 mM calcium chloride as well as magnesium chloride till change of color.

2.7.2. Binding capacity studies with SEDDS

AA loaded SEDDS (F₃-AA) were evaluated for their binding capacity towards calcium and magnesium ions. For this purpose, 0.01 M NaOH was used as dispersion medium by adjusting pH to 8.5 using 0.01 M HCl. SEDDS F₃-AA as listed in Table 1 was diluted from 1:10 up to 1:50 in dispersion medium and 5 mL of EBT solution was added to SEDDS dilutions as an indicator. Subsequently, 500 mL of each SEDDS was titrated with 10.0 mM calcium chloride and magnesium chloride till change of color indicating the end point.

In order to determine the amount of agaric acid remaining in SEDDS and being released into the medium, SEDDS (F₃-AA) dilution of 1:10 in 1 mL of 0.01 M NaOH was dialyzed against 10 mL of same medium using dialysis membrane of 20 kDa for 24 h. During incubation period, agaric acid being released from SEDDS diffused across the membrane into the acceptor medium. After incubation, the acceptor medium was titrated as described above for agaric acid content.

2.8. Growth inhibition studies

Primarily, AA loaded SEDDS (F₃-AA) were studied for their ability to inhibit bacterial growth using a model germ. E. coli was cultured in LB medium culture with a bacterial concentration of 0.5 McFarland. Inoculum was prepared by mixing 100 μL of bacterial suspension with 1.9 mL of LB medium. Different ratios of SEDDS F₃-AA and F₃ with and without AA, respectively were diluted (1:10 to 1:70) in 450 μL of growth medium (50% LB medium) and 50 μL of diluted bacterial suspension was added. In a second experiment, SEDDS dilutions 1:100, 1:200 and 1:400 of F₃-P (PMB HIPs), F₃-AA (AA) and F₃-PAA containing combination of PMB HIPs and AA were mixed in 450 μL of growth medium and 50 μL of 0.5 McFarland bacterial suspensions without dilution. Solutions of PMB and AA in growth medium were employed as negative control whereas diluted SEDDS without PMB and AA served as positive control. Afterwards, samples were incubated using thermomixer at 37 °C, 600 rpm for 20 h. 100 μL aliquots from each dilution were transferred to 96 well plate and measured for optical density using microplate reader at 600 nm. 500 μL of growth medium without SEDDS containing inoculum was taken as 100% for calculating percentage growth while growth medium without E. coli served as blank [19].

2.9. Statistical analysis

The results are compiled and expressed as means (±SD) of three experiments. The data obtained from the experiments were analyzed by using Student's t-test with 95% confidence interval (p value ≤0.05).

3. Results and discussion

3.1. Hydrophobic ion pairing and precipitation efficiency

HIPs of PMB were formed by using anionic agaric acid as lipidizing agent. PMB bears five primary amino groups imparting a cationic character while AA exhibits an anionic character because of its three carboxylic acid groups as shown in Fig. 1. The interaction between the positively charged PMB and negatively charged AA resulted in the formation of stable HIPs. Ion pair formation was evaluated in terms of precipitation efficiency as illustrated in Fig. 2. AA was added in increasing molar ratios to PMB in order to form HIPs. At stoichiometric molar ratio of 1:2 (PMB:AA), PMB was almost entirely precipitated from the solution. A further increase in molar ratio decreased the precipitation efficiency due to the formation of micelles. This effect is known from numerous other studies and has recently been summarized in a review [20]. In order to develop SEDDS for further studies PMB HIPs formed at stoichiometric ratio of 1:2 were used.

Log P<sub>bo-butanol/water</sub> of HIPs was determined showing the increase in lipophilicity due to HIPs formation. Lipophilicity of HIPs is crucial for their incorporation in SEDDS as well as their permeability across cell membranes [21]. The determined log P value of PMB HIPs was 1.7 ± 0.07 in comparison to PMB log P of −4.5.

3.2. Intermolecular interaction of PMB HIPs

A loss of AA carboxylic peak at 1686 cm<sup>−1</sup> might indicate ionic interactions between NH<sub>3</sub> + groups of PMB and carboxyl groups of AA or it can also be explained as a shift of the carboxylic acid peak from higher wavelengths to lower wavelengths of carboxylate ions towards PMB peak region in HIPs (Fig. 3).

3.3. ζ potential of HIPs

The change in ζ potential during HIP is an important parameter indicating the shift in surface charge of HIPs particle at the molecular level. The ζ potential of HIPs having been formed between PMB and AA is
Results demonstrate a shift in ζ potential from positive to negative with an increasing proportion of AA in HIPs. ζ potential shifted from +24 mV to 3.8 mV in case of stoichiometric ratio of 1:2 (PMB:AA). At stoichiometric ratio of 1:≥3 the ζ potential of HIPs was negative. Positively charged antibiotics such as polymyxin B are poorly absorbed because of the formation of insoluble ion pairs with endogenous anionic compounds before reaching the epithelial membrane [22]. In addition, cationic antibiotics show low mucus permeating properties due to ionic interactions with anionic mucus substructures such as sialic acid and sulfonic acid groups [23]. Furthermore, cationic antibiotics are trapped by anionic exopolysaccharides on the surface of biofilms so that they cannot reach the bacterial cell membrane [24]. These ionic interactions of antibiotics with all these endogenous anionic compounds limit their clinical application for treating infections. HIP formation can mask most of the cationic substructures on antibiotics avoiding such ionic interactions with endogenous anionic compounds. Once having reached the target germ, the charge of ion pairs has also a great impact on permeability across the cell membrane. Improved cell membrane permeability of ion paired hydrophilic ionizable drugs has been reviewed by Neubert [25].

3.4. Dissociation of HIPs

Mechanistic studies on PMB HIPs were conducted to investigate the impact of pH and ionic strength on HIPs dissociation at different time points. Dissociation of PMB HIPs at different pH and ionic strength is shown in Fig. 5. The results are in agreement with a previous study of Hetényi et al. indicating higher dissociation of HIPs with increasing pH and ionic strength of the released medium [14]. The stability of HIPs also influences the drug release from the oily
droplets of SEDDS. Yuan et al. described that stability of HIPs contributes to a more sustained drug release [26]. Dissociation of HIPs can also be explained on the basis of charge ratio between drug and lipidizing agent. HIPs formed by lipidizing agents having a monovalent counter ion quickly dissociate in comparison to HIPs formed by multivalent counter ions. The residual counter ion remains associated with HIPs via tail-tail hydrophobic interactions. These HIPs will eventually have a large hydrophobic surface area serving as a barrier that slows the diffusion of drug [20]. However, presence of endogenous anions in biological fluids such as phosphatidylserine, phosphatidylinositol, sialic acid or bile acids make in vivo conditions more complex effecting the stability of HIPs after their release from SEDDS. Moreover, dissociation of HIPs depends on the ionic strength of the medium having a substantial impact on drug release. Results showed that PMB HIPs dissociate more rapidly in media of high ionic strength. Additionally, long hydrocarbon chains of lipidizing agents also minimize the release by increasing hydrophobic interactions inside SEDDS and decreasing HIPs solubility in aqueous media. Bonengel et al. reported that highly stable HIPs strongly limit drug release whereas less stable HIPs causes extensive drug release [27]. The drug release from SEDDS is controlled by a simple diffusion process until equilibrium between the lipophilic phase of SEDDS and the aqueous phase of the release medium is reached [17]. The maximum solubility of HIPs in the SEDDS preconcentrate and in release medium termed as log $D_{SEDDS/RM}$ is therefore used to characterize drug release from SEDDS. The determined log $D_{SEDDS/RM}$ value of PMB HIPs with AA was 2.2.

3.5. SEDDS evaluation

In this study, SEDDS were developed and assessed with respect to their emulsifying properties and ability to entrap PMB HIPs and AA. To guarantee a short emulsification time the amount of surfactants in the formulations was adjusted to 60% (v/v). Additionally, macrogolglycerol hydroxystearate and macrogolglycerol ricinoleate also served as solvent for AA. Furthermore, glycerol monocaprylocaprate and DMSO served as the most suitable solvents for PMB HIPs. The amount of PMB HIPs incorporated in SEDDS was 0.312 mg/mL. The maximum payload of PMB HIPs was 5 mg/mL of SEDDS. As a matter of fact, payload of drugs mainly depends on their solubility in excipients used to constitute SEDDS. Griesser et al. reported variable payloads of same peptides in different SEDDS formulations [16].

As cytotoxicity assays with SEDDS are frequently performed on Caco-2 cells, this cell line was chosen for comparison reasons. As depicted in Fig. 6, increasing concentrations of SEDDS from 0.1 to 0.5% (v/v) did not cause a drop in cell viability below 80% over a period of 4 h [28,29]. However, 1% (v/v) SEDDS (FA-AA and FA-PAA) showed reduced cell viability suggesting AA concentration dependent toxicity of SEDDS. This effect might be at least to some extent attributed to presence of AA that can interact with cell membrane resulting in decreased viability of Caco-2 cells.

![Fourier-transform infrared spectra of polymyxin B (PMB), agaric acid (AA) and polymyxin B hydrophobic ion pairs (PMB HIPs).](image-url)
3.6. Chelation of AA and F₆-AA SEDDS

According to the results 1 mol of citric acid binds 38.43 ± 1.60 g of calcium and 23.70 ± 0.50 g of magnesium per mole of citric acid whereas 37.39 ± 0.92 g of calcium and 22.50 ± 1.01 g of magnesium is bound to 1 mol of AA. Citric acid and AA showed no significant difference in binding metal ions. As AA is an amphiphilic molecule, its hydrophilic tricarboxylic acid head groups likely accumulate on the surface of SEDDS being oriented towards the aqueous phase. These head groups in turn impart chelation properties to SEDDS as confirmed by results shown in Fig. 7(b). It was also observed that SEDDS have lower capacity to bind metal ions in comparison to pure AA in aqueous solution. The log Po of AA calculated to be 6.7 [30] might be the most plausible explanation for lower metal binding capacity of SEDDS. Because of this high log P, likely not all hydrophilic head groups of AA were accumulated on the surface of SEDDS. The results from SEDDS dialysis and titration revealed that 7% of AA were released into the aqueous medium while 43% remain on the surface of SEDDS and 50% remain inside SEDDS.

3.7. Antimicrobial activity

Divalent cations such as magnesium and calcium play an important role in mediating many cellular functions comprising ion homeostasis, signaling pathways and morphogenesis in microbial cells. Each divalent cation has a specific cellular function and its absence severely effects cellular mechanisms of microorganisms. In particular, calcium is an important element needed to synthesize bacterial exoskeleton for bacterial growth [31]. Calcium initiate polymerization of tubulin proteins to form bacterial cell wall [32]. In the absence of calcium, bacterial cells are unable to form their cell wall and become spherical with swollen large vacuoles that ultimately results in cell lyses [33]. Magnesium maintains integrity of bacterial cell membrane and is also considered a vital element for promoting microbial enzymatic activity [34]. Magnesium deficiency results in stunted enzymatic activity by halting vital
biological functions of bacterial cells [35]. Graham et al. reported about the inhibition of bacterial growth by tricarboxylic acid that was attributed to the chelation of metal ions apart from their effect on pH [36]. Similar results have been stated by Booth and Kroll showing that tricarboxylic acid possess greater affinity for divalent cations than the components of cell membrane resulting in its destabilization [37]. This chelation based destabilization changes bacterial membrane permeability by releasing lipopolysaccharides [38]. In the present study, lipophilic tricarboxylic acid (AA) was incorporated into SEDDS and studied for growth inhibition. Briefly, AA containing SEDDS FA-AA displayed remarkable inhibition of E. coli growth as shown in Fig. 8. The difference in growth inhibition of E. coli was in accordance with the applied concentrations of AA. SEDDS (FA-AA) halted the growth of E. coli below 20% in dilution range of 1:10 and 1:20. Moreover, SEDDS containing lower concentrations of AA at higher dilutions resulted in slow growth of E. coli. Furthermore, SEDDS (FA-AA) having been diluted 1:70 inhibited growth between 40 and 45%. Results confirm that the concentration of AA in SEDDS has a direct impact on the growth inhibition of E. coli as also previously reported about different drug delivery systems conjugated with tricarboxylic acid [5]. Debbabi et al. reported a pronounced antimicrobial activity of chitosan conjugated to a tricarboxylic acid against E. coli [39]. In addition to growth inhibiting properties, chelating agents were reported to increase efficiency of antimicrobials [40]. This combination, however, requires a delivery system that can keep both antibiotics and chelating agents together. Otherwise these components will mislay in a vast biological system without providing any benefit. Previously, a combination was made by Angelo et al. in form of nanoparticles by forming hydrophobic ion pairs of chlorhexidine with losartan for treatment of endocarditis [41]. Unfortunately, ion paired nanoparticles soon dissociated and separated in aqueous medium. For stability of HIPs, SEDDS are considered more stable systems due to low dielectric constants of the excipients used. Therefore, SEDDS were chosen as appropriate delivery system for combination of PMB and AA to reach microbes in a synchronized way for enhanced antimicrobial effect. SEDDS FA-PAA containing combination of PMB and AA were studied for antibacterial activity and indeed showed a marked improvement in antimicrobial activity as can be seen in Fig. 9. FA-PAA showed 36- and 13-fold higher microbialic effect than FA-AA and FA-P, respectively. These SEDDS are designed to be applied locally providing an intimate contact with pathogens present on mucosal surfaces such as the intraoral mucosa. AA present in SEDDS acts as a lipophilic derivative of citric acid binding divalent cations and improving the permeation of PMB across bacterial membranes that eventually results in enhanced antimicrobial activity. Likewise, a combination of a lipophilic derivative of ethylenediaminetetraacetic acid (alkyl-EDTA) as chelating agent was incorporated in combination with chlorhexidine in SEDDS for enhanced antibacterial effect [19]. Similar findings had been reported which backed the capabilities of chelating agents to augment the bactericidal activity of a number of antimicrobial agents [40,42].

4. Conclusion

In the present study PMB was ion paired and incorporated into SEDDS. Stability of the PMB HIPs was assessed by evaluating dissociation in different release media at different pH and ionic strength over time. Chelating properties of SEDDS were determined by assessing capacity to bind calcium and magnesium. SEDDS containing PMB and AA was assessed for their enhanced antimicrobial activity. Results suggested that dissociation of PMB HIPs was highly depended on pH and
ionic strength. SEDDS exhibited chelation properties due to presence of the hydrophilic head groups of AA on their surface. The combination of PMB and AA in SEDDS showed enhanced antimicrobial properties against *E. coli*. Hence, AA proved to be a promising potentiating agent for enhancing efficiency of antibiotics against microbes.

**List of abbreviation**

| Abbreviation | Description                          |
|--------------|--------------------------------------|
| AA           | Agaric acid                          |
| CaCl₂        | Calcium chloride                     |
| DMSO         | Dimethyl sulfoxide                   |
| *E. coli*    | Escherichia coli                     |
| FA-AA        | SEDDS containing AA                  |
| FA-P         | SEDDS containing PMB HIPs           |
| FA-PAA       | SEDDS containing PMB HIPs and AA    |
| HIPs         | Hydrophobic ion pairs                |
| MgCl₂        | Magnesium chloride                   |
| PMB          | Polymyxin B                          |
| SEDDS        | Self-emulsifying drug delivery systems |

**CRediT authorship contribution statement**

Aamir Jalil: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. Mulazim Hussain Asim: Investigation, Validation. Imran Nazir: Investigation, Validation. Barbara Matuszczak: Formal analysis, Software. Andreas Bernkop-Schnürch: Supervision, Conceptualization, Writing - review & editing.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Fig. 8.** Percentage growth of *E. coli* after 20 h of incubation with agaric acid loaded SEDDS (white bars) and unloaded SEDDS (black bars) used as control in indicated dilutions of 1:10 (3000 μg/mL), 1:20 (1500 μg/mL), 1:30 (1000 μg/mL), 1:40 (750 μg/mL), 1:50 (600 μg/mL), 1:60 (500 μg/mL) and 1:70 (428.6 μg/mL). All values are mean ± SD of three experiments.

**Fig. 9.** Percentage *E. coli* growth in presence of SEDDS dilution 1:100, 1:200 and 1:400 of Fₐ-AA containing agaric acid (300 μg/mL, 150 μg/mL and 75 μg/mL) and Fₐ-P having PMB (3.125 μg/mL, 1.56 μg/mL and 0.78 μg/mL), respectively while Fₐ-PAA SEDDS dilutions containing combination of similar concentrations depicting augmented antimicrobial effect. All values are mean ± SD of three experimental values.
