Antimicrobial activity of self-assembled structures formed by protected amino acids

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Abstract: Herein we report the antimicrobial activity of the self-assembled structures formed by protected single amino acids (SAAs) and also their application as a tool for overcoming antibiotic resistance. Interestingly, SAAs modified with simple protecting groups like -Fmoc, -Boc, -Cbz exhibit antibacterial activity and is useful in overcoming the antibiotic resistance in wide spectrum of bacterial strains namely *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Psedomonas aeruginosa, Vibrio cholera and Enterococcus faecalis* as in there presence the concentration of antibiotic required to inhibit bacterial strain was decreased manifold . The SAAs themselves exhibited antimicrobial activity albeit higher doses of the SAAs as compared to chloramphenicol alone was required for inhibition. However, in presence of antibiotics the concentration required for SAAs as well as antibiotic was lowered manifold particularly in case chloramphenicol of *S. Aureus, P.aeruginosa* and *E. Faecalis* the IC50 value of peptides alone was even less than chloramphenicol. When peptides were used in combination with chloramphenicol antibiotic resistance was overcome in wide spectrum of bacterial strains and the concentration of chloramphenicol required was drastically reduced.

1. Introduction

Antibiotic resistance is one of the most challenging problem to fight against microbial diseases which occur in humans and animals.[1] Excessive use of antibiotics is not safe and recommended as they can inhibit good microorganisms which are vital for the existence. Wide spectrum of Gram-positive and -negative bacteria that cause plethora of microbial infections are becoming more resistant to commonly used antibiotics.[2] The most commonly reported bacterial strains which develop antibiotic resistance are *E. coli, S aureus, S.*

pneumoniae, and K. pneumoniae according to recent data given by World Health Organization,

Antibiotics also have their own side-effects and the clinicals usually do not recommend use of antibiotic unless there is no alternative. Moreover, it is also imperative that antibiotics are made effective to stop infection at very low concentration, since a higher dose of antibiotic will also inhibit the useful microfauna required for essential processes inside the body along with xenobiotic toxicity of course. Hence, it is of utmost importance to find novel strategies which could overcome the global problems of antibiotic resistance. In this context antimicrobial peptides and modified amino acids serve as excellent bio-organic scaffolds, since they are biocompatible and known to possess antimicrobial activity as they can penetrate inside the cell membrane of bacteria causing its disruption. There are many literature reports wherein Fmoc modified single amino acids/ short peptides has been used as antibacterial agents.[3]The antibacterial activity of amino-acid based hydrogels and Fmoc-Phenylalanine is particularly well reported. In this context,Chen and co-workers prepared a hydrogel by the co-assembly of Fmoc-phenylalanine and Fmoc-leucine which revealed selective antimicrobial action against Gram positive bacterial without causing any toxicity in mammalian cells, thus making it useful for the design of anti-microbial coatings in hospitals.[4]

In another work Xie et al. reported the influence of nanostructured morphology of the hydrogels formed by Fmoc-tryptophan (Fmoc-W), Fmoc-methionine (Fmoc-M), and Fmoc-tyrosine (Fmoc-Y) on the antibacterial activity and illustrated more compact, rigid and aligned nanofibers exhibit more antimicrobial potency.[5] Further the same research group illustrated antibacterial activity Fmoc-Leucine/Fmoc-Lysine hydrogel, against Gram-positive *S. aureus*, *B. subtilis*, *L. monocytogenes*, and Gram-negative *E. coli*, *Sh. sonnei*. They found that the hydrogel was composed of twisted nanobelts the antibacterial activity of hydrogels enhances with the prolonged incubation time.[6] Disulfide-linked nanospheres exhibited antibacterial efficacies in a range of Gram-positive and Gram-negative bacteria was reported by Ahmed and co-workers.[7] In yet another interesting research Thakur and coworkers reported the antibacterial activity of Fmoc-phenylalanine (Fmoc-F) both in hydrogel and solution phases as it possess surfactant like properties due to the bacterial cell membrane is ruptured.[8]

Further to make Fmoc-F potent againg Gram negative bacterial they prepared a formation of Fmoc F Gram-negative specific antibiotic aztreonam (AZT). This formulation displayed antibacterial activity against both Gram-positive and Gram-negative bacteria and produced a synergistic effect and higher efficacy against *P. aeruginosa* due to the increased Fmoc-F permeability by Thakur and co-workers.[9] A novel hybrid hydrogel with aggregation-induced emission behavior was derived by Jia and co-workers, where self-assembly of F-moc and berberine chloride possessing antibacterial and anti-biofilm activity against Gram-positive and Gram-negative bacteria was discussed.[10] Zhao and co-workers reported the development and characterization of synthetic peptides with antibacterial activity against *Pseudomonas aeuruginosa* and *Staphylococcus aureus*.[11] In one of the work, Nanda et al. synthesized Fmoc-derivatives of three analogues which formed hydrogels possessing different gelation efficacy and kinetics. The derivatives have shown promising antimicrobial activity against gram-positive bacteria.[12] In a recent report Wangoo and co-workers designed Fmoc-phenylalanine (Fmoc-F)-based hydrogels using trisodium citrate as a pH modulator. Interestingly the hydrogels prepared by this method exhibits antibacterial action against both Gram-positive and Gram-negative bacteria.[13] Similar research was carried out by Zhong and co-workers. They developed F-moc conjugated amino acids as promising antibacterial agents with self-assembly capabilities.[14]

As compared to other commonly used antibiotics in market, chloramphenicol is more effective in curing bacterial infection since it is less prone to antibiotic resistance.[15] In one of the work, G.K. et al. described the possible role of the p-NO_2 group of chloramphenicol in aplastic anemia. However, excessive use of chloramphenicol is associated with aplastic anaemia.[16] In this study, we have used single amino acids modified with protecting group as a methodology to overcome chloramphenicol resistance in bacteria and also to assess the antibacterial properties of modified single amino acids as such. It was noted that in presence of protected amino acids the concentration of chloramphenicol required to inhibit the bacterial growth was drastically decreased. These results are very important and illustrate potential application of protected amino acids as an efficient antimicrobial agent.

2. Results and discussion

In this study**,** we aimed to use the self-assembling properties of protected amino acids and its complexation with antibiotics as a tool to overcome the antibiotic (chloramphenicol)

resistance. From the literature overview it is evident that nanostrucured morphologies formed by modified amino acids/ peptides self-assembly plays a crucial role in imparting antibacterial action to the compound. Hence, we started our experiments by first studying the self-assembling properties of protected amino acids alone in 1% DMSO:water. We used twelve protected amino acids namely Fmoc-Threonine (Fmoc-Thr), Fmoc-Proline (Fmoc-Pro), Fmoc-Serine (Fmoc-Ser), Fmoc-Aspartic acid (Fmoc-Asp), Fmoc-Isoleucine (Fmoc-Ile), Fmoc- Cysteine (Fmoc-Cys), Fmoc- Methionine (Fmoc-Met), Boc-Glutamine (Boc-Gln), Boc-Lysine (Boc-Lys), Boc-Valine (Boc-Val), Cbz-Trp and Cbz-Tyr. The self-assembling properties of twelve amino acids with and without antibiotic are summarised in Table 1.

Table1. Self-assembly studies of protected amino acid alone with and with chloramphenicol.

S.No.	**Amino acids**	**Morphology (simple com.)**	**Morphology (simple + anti)**
1.	Fmoc- Threonine	Droplet + Sphere	Changed
2.	Fmoc- Proline	Sphere + fiber	changed
3.	Fmoc- Serine	fiber	unchanged
4.	Fmoc- Aspartic acid	Fiber capsual	unchanged
5.	Fmoc- Isoleucine	Fiber	unchanged
6.	Fmoc- Cysteine	fiber	Changed (less fiber)
7.	Fmoc- Methionine	nothing	unchanged (little fiber)
8.	Boc- Glutamine	Fiber	Changed
9.	Boc- Lysine	Nothing	unchanged
10.	Boc- Valine	Sphere	Changed

11.	Cbz- Tryptophan	nothing	unchanged
12.	Cbz- Tyrosine	No morphologies	Changed (little fiber)

From the twelve SAAs we studied we could observe favourable assemblies and morphological transitions as observed through optical microscopy in six conjugates namely Fmoc-Threonine , Fmoc-Proline, Fmoc-Cystine, Boc-Valine, Boc-Glutamine and Cbz-Tyrosine.

The chemical structure of Fmoc-Threonine , Fmoc-Proline, Fmoc-Cystine, are shown in Figure 1a, 1b and 1c respectively. From the optical microscopy image it can be surmised that Fmoc-Thr assembles to globular structures (Figure 1 d) which change to fibril and micellar structures (Figure 1 g), Fmoc-Pro assembles to spheres (Figure 1e) which change to globullar structures (Figure 1h) and Fmoc-Cys assembles to crystal like assemblies (Figure 1 f) to thin fibers (Figure 1 i).

The chemical structure of Boc-Valine, Boc-Glutamine and Cbz-Tyr are shown as Figure 2, 2b and 2c respectively. The optical microscopy images of **Boc-Val** reveals small micelles (Figure 2 d) which appear to change to globular structures (Figure 2 g) on interaction with chloramphenicol. Boc- Gln assembles to thin fiber (Figure 2 e) which change to thick fibrils and small micelles (Figure 2 h). Cbz-Tyr assembles to thin small fibers (Figure 2 f) which change to thin long fibers and micelles after interaction with chloramphenicol (Figure 2 i).

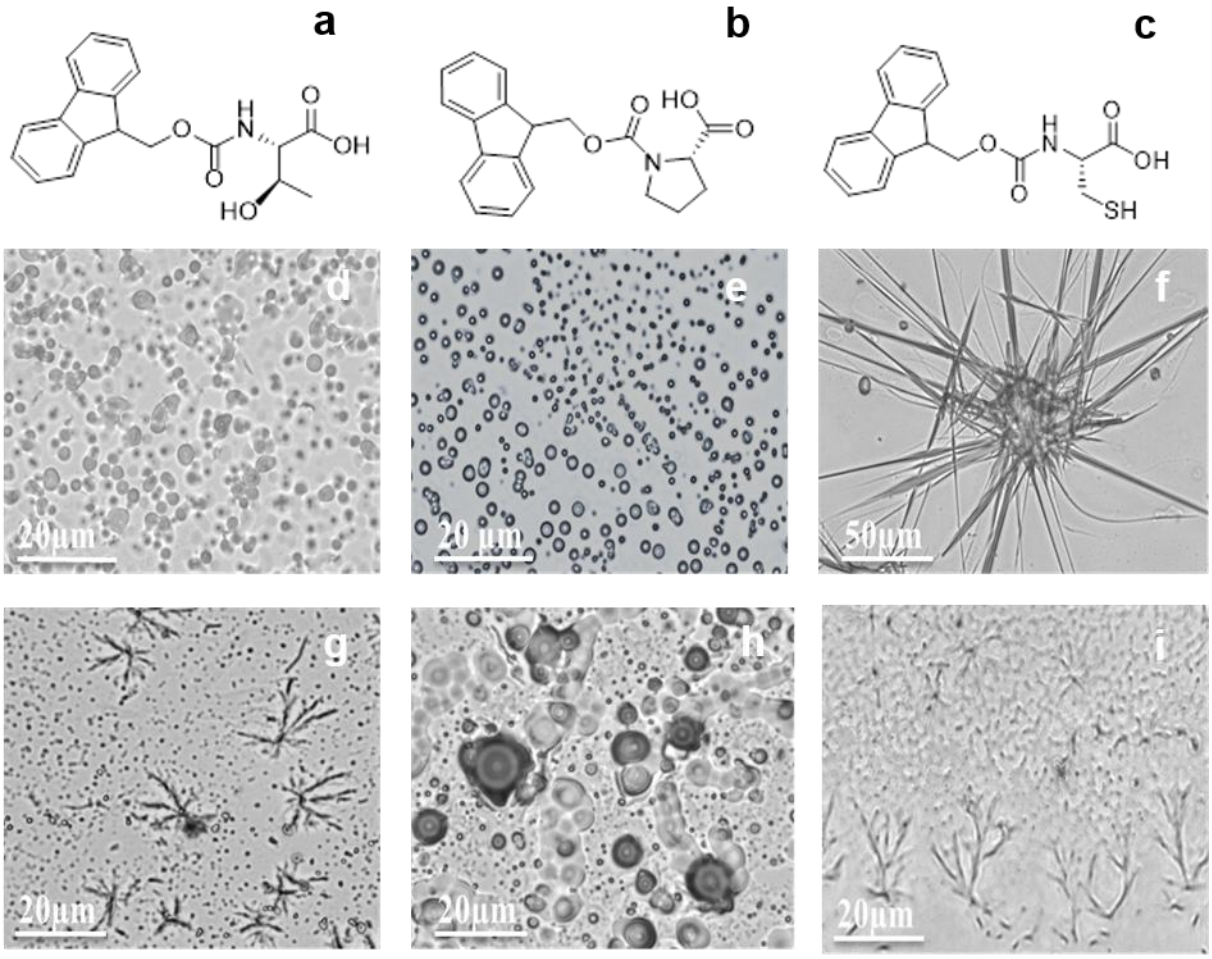

Figure: 1 (a) Chemical strucutre of **Fmoc-Thr**; (b) Chemical structure of **Fmoc-Pro**; (c) Chemical structure of **Fmoc-Cys**; (d) Optical microscopy image of **Fmoc-Thr** alone**;** (e) Optical microscopy image of **Fmoc-Pro** alone; (f) Optical microscopy image of **Fmoc-Cys**; (g) Optical microscopy images of **Fmoc-Thr** with antibiotic; (f) Optical microscopy image of **Fmoc-Pro** with antibiotic; (i) Optical microscopy image of **Fmoc- Cys** with antibiotic.

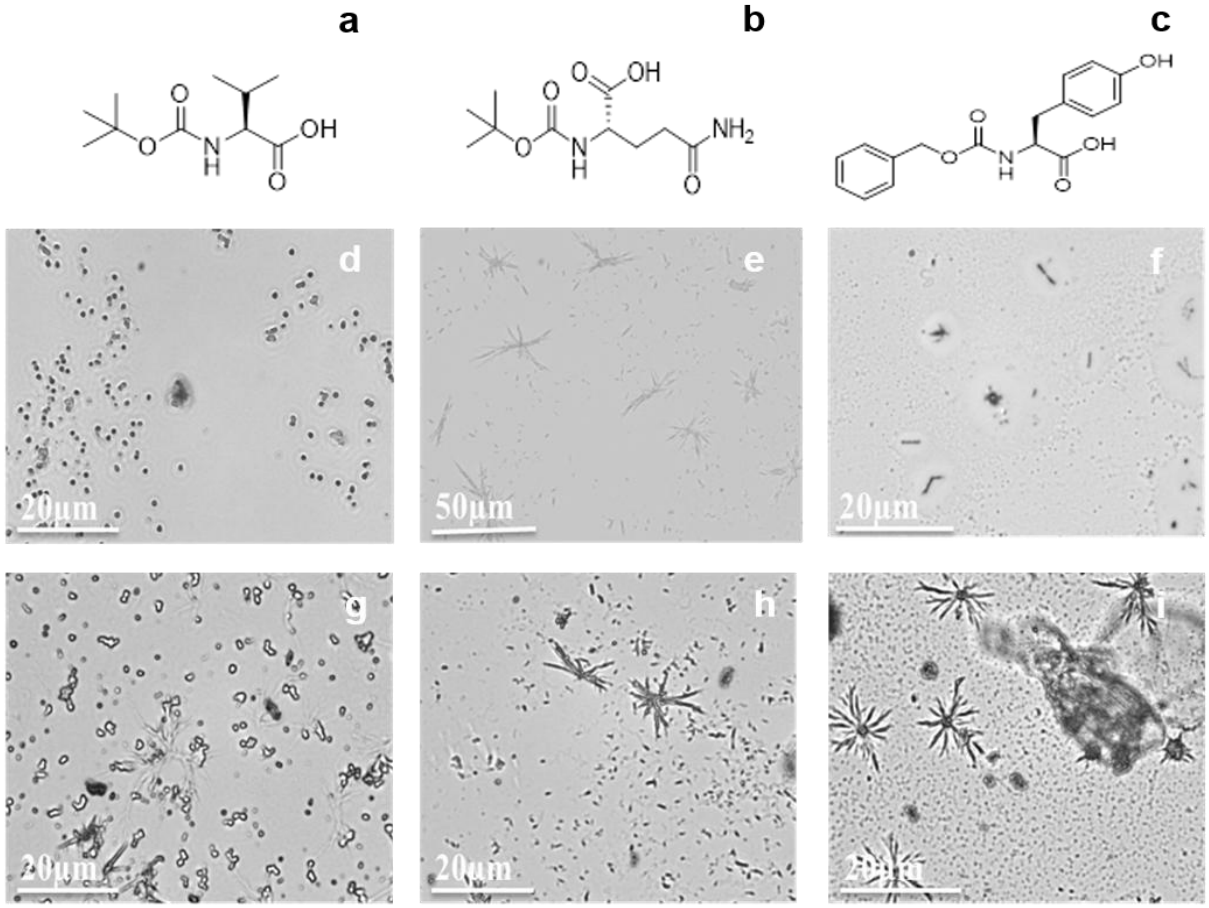

Figure: 2 (a) Chemical structure of **Boc- Val**; (b) Chemical structure of **Boc- Gln**; (c) Chemical structure of **Cbz- Tyr**; (d) Optical microscopy image of **Boc-Val** alone; (e) Optical microscopy image of **Boc-Gln** alone; (f) Optical microscopy image of **Cbz-Tyr** alone; (g) Optimal microscopy image of **Boc-Val** with antibiotic; (h) Optimal microscopy image of **Boc-Gln** with antibiotic; (i) Optimal microscopy image of **Cbz-Tyr** with antibiotic.

Since, the optical microscopy images suggested favourable complexation of the six SAAs with chloramphenicol, we studied the antibacterial properties of these SAAs alone and in presence of chloramphenicol. The antibacterial properties of Fmoc-Thr, Fmoc-Pro and Fmoc-Cys alone and in presence of chloramphenicol are present in Table 1. The IC50 value of Fmoc-Thr was found to be 250 µg/ml for all six strains *E.coli, S.aureus, B.subtilis, P. aeruginosa, V. cholera* and *E. feacalis.* The IC50 concentration of antibiotic chloramphenicol on the other hand for these strains was 3.95µg/ml, 125µg/ml, 3.95µg/ml, 125 µg/ml, 31.25 µg/ml, and125 µg/ml respectively. However, when Fmoc-Thr/Chloramphenicol complex was used the concentration of antibiotic required to inhibit the bacteria was drastically reduce for *S. aureus* as against 125µg/ml of chloramphenicol only 3.95µg/ml of chloramphenicol is now required to achieve IC50. The concentration of Fmoc-Thr required to achieve IC50 was also reduced from 250 µg/ml to 62.5 µg/ml. Similarly for *P.aeruginosa* and *E. faecalis* the concentration of chloramphenicol required to achieve IC50 was reduced from 125µg/ml to 15.6 µg/ml and 3.9 µg/ml respectively. Hence Fmoc-Thr was efficient in overcoming antibiotic resistance and can be potentially used to cure these infections as combination therapy.

Table 1: Antibacterial activity of Fmoc-Thr, Fmoc-Pro and Fmoc-Cys.

Strain	**Escherichia coli**	**Staphylococcus aureus**	**Bacillus subtilis**	**Psedomonas aeruginosa**	**Vibrio cholerae**	**Enterococcus faecalis**
SAAs/ chloermphenicol/ complex	**IC 50 (µg/ml)**					
Fmoc-Thr	**250**	**250**	**250**	**250**	**125**	**250**
Fmoc-Pro	**125**	**250**	**125**	**125**	**125**	**250**
Fmoc-Cys	**250**	**250**	**250**	**250**	**125**	**250**
Fmoc-Thr/ Chloramphenicol	**125/7.8**	**62.5/3.9**	**62.5/3.9**	**250/15.6**	**250/15.6**	**62.5/3.9**
Fmoc-Pro/ Chloramphenicol	**15.6/0.9**	**31.2/1.9**	**31.25/1.9**	**125/7.8**	**62.5/3.9**	**31.25/1.9**
Fmoc-Cys/ Chloramphenicol	**125/7.8**	**62.5/3.9**	**125/7.8**	**250/15.6**	**125/7.8**	**62.5/3.9**

Chloramphenicol	**3.95**	**125**	**3.9**	**125**	**31.25**	**125**

Similarly, the antibacterial action of Fmoc-Pro was studied in six strains using its varying concentration alone and in combination with chloramphenicol. Fmoc-Pro revealed even better antibacterial properties than Fmoc-Thr. In the presence of Fmoc-Pro, the concentration of chloramphenicol required to inhibit the bacterial strains was reduce with the IC 50 value of chloramphenicol required to inhibit *E.coli* reducing from 3.95μg/ml (antibiotic alone) to 0.9 μg/ml for Fmoc-Pro/ chloramphenicol complex. The antibiotic was again very effective in three strains *S. Aureus, P.aeruginosa* and *E. faecalis* where 125 μg/ml of chloramphenicol was required to inhibit 50% bacterial strains. In presence of Fmoc-Pro this value decreased to 1.9, 7.8 and 1.9 μg/ml respectively. Hence, the antibiotic resistance was efficiently overcome by this SAA too. Similar observations were noted for other SAAs Fmoc-Cys, Boc-Gln, Boc-val and CBz-Tyr which are summarised in Table 1 and Table 2. It can be surmised that complexation of antibiotic with SAAs reduced IC50 of SAAs as well as antibiotic in majority of strains.

Table- 2 Antibacterial activity of Boc-Gln, Boc-Val and CBz-Tyr

Strain	***Escherichia coli***	***Staphylococcus aureus***	***Bacillus subtilis***	***Psedomonas aeruginosa***	***Vibrio cholerae***	***Enterococcus faecalis***
Drug	Inhibition concentration					
Boc- Gln	250	250	125	250	125	125
Boc-Val	250	250	125	125	62.5	125
CBz-Tyr	125	125	250	125	125	250

Boc-Gln/ Chlorophenicol	125/7.8	62.5/3.9	125/7.8	**250/15.6**	125/7.8	62.5/3.9
Boc-Val/ Chloramphenicol	31.25/1.9	15.6/0.9	31.25/1.9	125/7.8	62.5/3.9	62.5/3.9
CBz- Tyr/ Chloramphenicol	31.25/1.9	31.25/1.9	31.25/1.9	125/7.8	62.5/3.9	62.5/3.9
Chloramphenicol	3.95µg	125µg	3.9µg	125µg	31.25µg	125µg

Table 3 illustrate a summarised representation of the concentration of antibiotic required to inhibit six bacterial strains. It may be noted that in all strains the conjugates have enhanced the antibacterial property of antibiotic in comparison to antibiotic alone except for the inhibitory concentrations marked in Red. In case of *S. aureus* and *P. aeruginosa* in particular the antibacterial property of chloramphenicol has enhanced manifold. For example in case of *S. aureus* in presence of using **Boc-Val/Chloramphenicol** conjugate antibiotic required is only 0.9 µg as against 125µg which was required for antibiotic alone

Table 3. Antibiotic required for different strains in the presence of conjugate as compared to antibiotic alone.

Strain	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Psedomonas aeruginosa*	*Vibrio cholerae*	*Enterococcus faecalis*
Drug/ Drug-SAAs	**Inhibition concentration of antibiotic in µg/ml in presence of SAAs**					
Chloramphenicol	**3.95µg**	**125µg**	**3.9µg**	**125µg**	**31.25µg**	**125µg**
Fmoc-Thr/ Chloramphenicol	7.8	3.9	3.9	15.6	15.6	3.9
Fmoc-Pro/ Chloramphenicol	0.9	1.9	1.9	7.8	3.9	1.9
Fmoc-Cys/ Chloramphenicol	7.8	3.9	7.8	15.6	7.8	3.9
Boc-Gln/ Chlorophenicol	7.8	3.9	7.8	15.6	7.8	3.9
Boc-Val/ Chloramphenicol	1.9	0.9	1.9	7.8	3.9	3.9
CBZ- Tyr/ Chloramphenicol	1.9	1.9	1.9	7.8	3.9	3.9

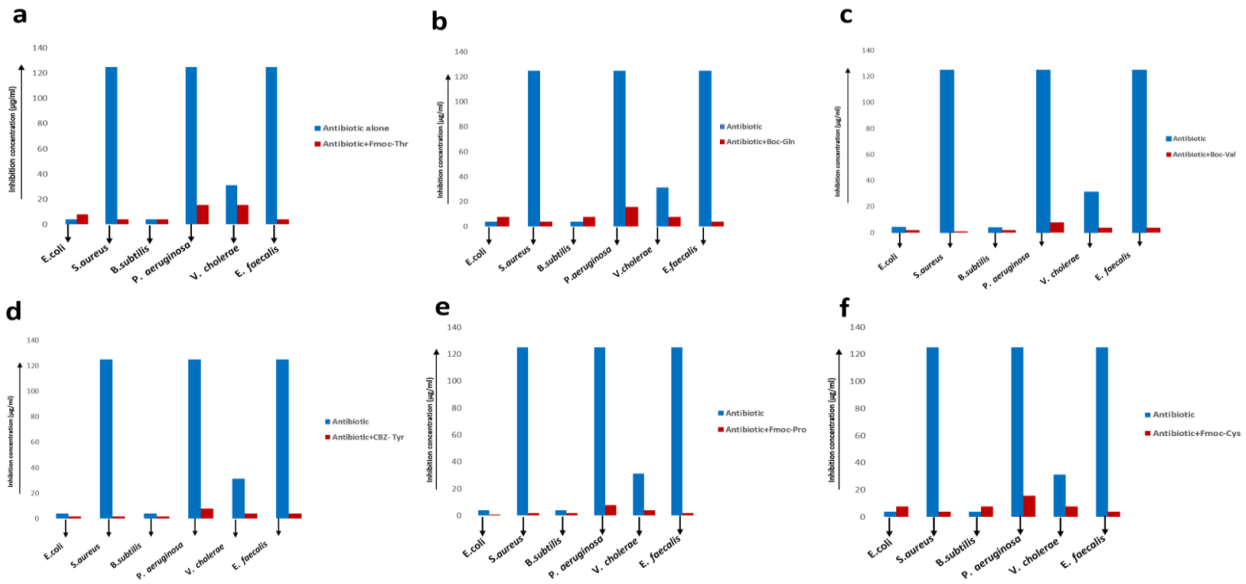

Figure 3: Antibacterial study graph of SAAs on six strains.

Conclusions

We have studied the antibacterial properties of protected single amino acids (SAAs) and assessed its application in antibiotic resistance against six bacterial strains *E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, *V. cholera* and *E. faecalis.* The self-assembling properties of these these SAAs and its complexation with chloramphenicol was studied through optical microscopy. Subsequently the SAAs which revealed morphological transition with chloramphenicol were tested for their antibacterial activity. The antibacterial studies suggest SAAs can be efficiently used for overcoming antibiotic resistance in bacterial strains. The IC50 value of chloramphenicol was drastically reduced in case of thre bacterial strains namely *S. aureus, P. aeruginosa* and *E. faecalis.* These results are significant as they illustrate important application of self-assembled amino acids as therapeutic tool against antibiotic resistance and its future implications as antimicrobial agents.

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