Self-Assembling, Ultrashort Peptide Gels as Antimicrobial Biomaterial

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Abstract: Supramolecular antimicrobial hydrogels based on peptides are attractive soft materials for the treatment of infections, considering their ease of preparation and benign fate in biological settings and in the environment. In particular, stimuli-responsive systems that can be assembled/disassembled *ad hoc* could offer the opportunity to switch on/off their bioactivity as needed. Besides, the shorter is the peptide, the lower its cost of production. However, a structure-to-function relationship is yet to be defined, and reported activities are generally not yet competitive relative to traditional antibiotics. Inspiration for their design can be found in host defense peptides (HDPs), which can self-assemble to exert their function. This article reviews research developments in this emerging area, and it examines features, differences and similarities between antimicrobial and amyloid peptides to open the avenue towards the next generation of supramolecular antimicrobial peptides as innovative therapeutic materials.

Keywords: peptides, self-assembly, antimicrobial, amyloid, hydrogel, smart materials.

1. INTRODUCTION

1.1 Towards a post-antibiotic era

In recent years, most pharmaceutical companies had severely reduced their R&D investments in new antimicrobial agents, because of the poor returns for antibiotics that were approved to market in the last decades [1]. By contrast, today, research towards new antimicrobial agents is reviving, in light of the worldwide emergency we are facing in terms of antimicrobial resistance (AMR). The term antimicrobial includes antibiotics, anti-viral and anti-malarial agents. The causes of AMR spreading are varied (Fig. 1), and they include misuse of such agents to treat humans and animals, especially in countries where antimicrobials use is not regulated and they are readily available without medical prescription. There is also the need for clear guidelines concerning the safe disposal of unused and expired antibiotics. This is important to reduce the amount of antibiotics that persist in the environment and could cause further spreading of AMR. The serious risk is that, if the current trend does not stop, we will soon be entering the "post-antibiotic" era, where simple infections could be again the leading cause of death for many humans worldwide [2].

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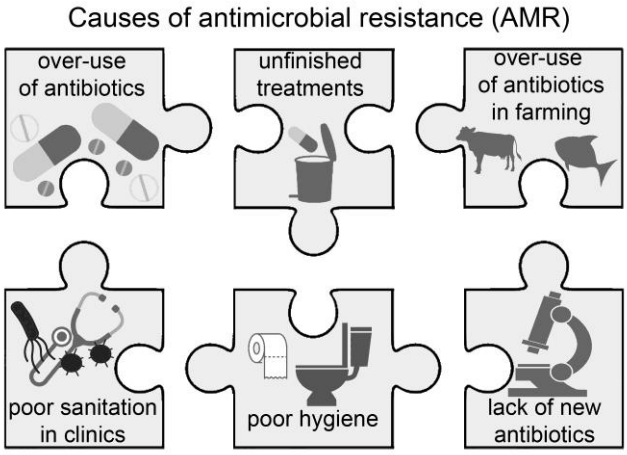


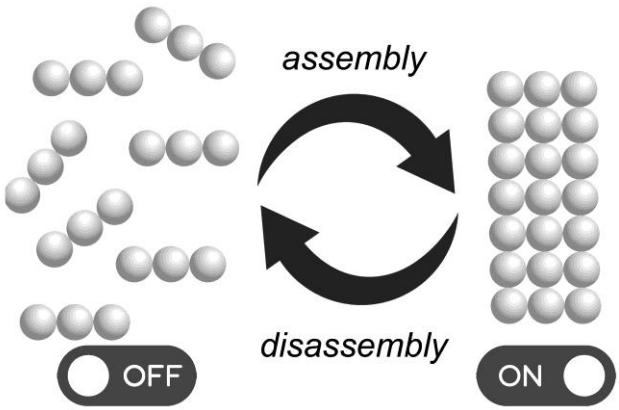
Fig.1. Common causes of antimicrobial resistance (AMR).

To circumvent the issue of AMR, research for new antimicrobials has been extended well beyond conventional drug molecules, to include also nanomaterials, such as nanoparticles [3] and peptide-derivative nanostructures [4]. In particular, the latter are attracting wide interest due to their inherent biocompatibility and the fact that endogenous antimicrobial peptides are widely known, and nature has shown they can be very effective against infections [5]. Peptides can easily be modified at the level of their primary sequence, for instance to introduce non-natural moieties that may extend their half-time *in vivo* and may also improve their activity [6-10]. Unfortunately, prediction of the effects of such modifications on their secondary and tertiary structure, and especially on their supramolecular organization, hence bioactivity, is very difficult to anticipate. This review will thus focus on recent developments in the research areas of antimicrobial peptides in common with amyloid peptides and self-assembling peptides that can help in the development of the next generation supramolecular, smart, antimicrobial systems.

1.2 Why peptide-based hydrogels

Hydrogels are soft materials that incorporate and retain a vast amount of water and are ideal candidates to mimic natural tissues, and especially the extracellular matrix. Therefore, they are highly attractive in the medicinal field for applications that span from tissue engineering, to wound healing, to advanced media for drug release [11]. They can be composed of chemical or physical networks made of a variety of molecules, such as synthetic polymers [12], polysaccharides [13], or protein/peptides [14]. They can also be designed to include nanomaterials and further improve their physico-chemical properties [15, 16]. Indeed, nanomaterials can introduce additional features not only for a quantitative, rather a qualitative leap in nanomedicine [17].

Amongst the various types of hydrogels, those composed of ultrashort peptides (*i.e.*, 2-3 amino acids) are particularly attractive, for a number of reasons. Firstly, peptides can easily be produced with a variety of methods, of which the most popular are chemical synthetic approaches for short sequences. It should be noted, though, that solid-phase approaches require a large excess of reagents and solvents, and generate a vast amount of waste. Secondly, costs increase exponentially with the length of the sequence, making amino acid derivatives, di- and tri-peptides amongst the more economically viable options. Thirdly, only such simple compounds can easily be produced in liquid-phase, at significantly lower cost [18]. Furthermore, short peptides are far more chemically robust than large proteins that require correct folding for bioactivity relying on non-covalent, weak interactions that can easily get disrupted [14]. In addition, as small molecules of synthetic origin, they allow for a higher and simpler level of



control over chemical exact composition, with very high batch-to-batch reproducibility, in contrast with proteins of natural origin

or other macromolecules. Finally, as small molecules, they pose significant lower risks of immune response relative to polypeptides and proteins.

Perhaps one of the most important reasons that gives a net advantage to supramolecular hydrogels over conventional macromolecular systems is the ability to use a simple molecule of exact chemical composition that can self-assemble into a macroscopic, dynamic material. Indeed, macromolecular hydrogels are often composed by several compounds of similar chemical nature; poor control over the molecular weight results in more heterogeneous systems that do not allow for precise control over their physico-chemical behavior. Additionally, hydrogels composed of covalent linkages require far more energy for their disruption and reformation. By contrast, self-assembling small molecules offer a simple means to achieve a macroscopic system of defined chemical composition that can be disrupted and reformed with a small energy requirement, since it is based on weak interactions. A system of this kind allows finer control over its formation and disassembly. As a result, supramolecular hydrogels can be envisaged as dynamic systems that can easily be switched between two states; *i.e.*, assembled (gel) and disassembled (sol). A plethora of different stimuli have been developed in recent years to trigger transformations of this kind in smart systems, and their description can be found elsewhere [19].

Stimuli-responsive systems are indeed attractive in light of the fact they can adapt to the environment and evolve over time, an ability typically found in living organisms. Importantly, in the context of AMR, the dynamic behavior can be envisaged as a means to control bioactivity. For instance, if a supramolecular system has antimicrobial activity only in its assembled state, then it could be "switched on" when needed to treat infections, then "switched off" to avoid release of antimicrobial agents in the environment (Fig. 2). In this manner, the antimicrobial activity would be exerted with spatiotemporal control exquisitely to address therapeutic needs.

We believe this avenue is highly promising for the treatment of infections, and could be envisaged not only for topical treatments, such as wound dressings and hydrogel applications on mucosae, but also for advanced formulations containing nanogels for oral administration. However, the way ahead is still long and full of challenges, therefore it requires collaborative efforts from different scientific areas and disciplines that could help in the design of new therapeutic solutions, as described further below.

Fig. 2. Assembly/disassembly of a small molecule can be used as a means to switch on/off a system if a certain function (*e.g.*, antimicrobial activity) lies only within the assembled state. As an example, tripeptide molecules (represented by three grey beads, on the left) can assemble into a functional supramolecular stack (right) and disassemble back to the initial state (left).

2. PEPTIDE SELF-ASSEMBLY

2.1 Ultrashort peptide self-assembly

Self-assembly of ultrashort peptides into supramolecular structures for hydrogelation has been the subject of intense studies, yet it is not completely understood. A direct correlation between peptide structure and self-assembly for the design of new gelators is currently lacking, although structural variation is known to have an important impact on self-assembly, and hydrophobicity stands out as a key requirement for molecular aggregation in water [20]. In a minimalistic search, Gazit and collaborators discovered that one [21] or two [22] units of phenylalanine are powerful self-assembling motifs towards supramolecular fibers. However, stable hydrogels are not obtained from the latter [23, 24], unless the dipeptide is cyclized to the corresponding 2,5-piperazinedione [24].

Another very useful approach to promote hydrogelation of the Phe-Phe motif [25] consists of adding rigid, protective groups at the peptide N-terminus that can template self-assembly and engage in aromatic interactions driving supramolecular organization (Fig. 3). Many examples of this approach can be found in the literature; the most popular moieties employed to this end are the fluorenylmethyloxycarbonyl (Fmoc) moiety or naphthalene (Nap) derivatives [26-27]. Recently, also heterocycles, such as indole or carbazole, proved effective as alternatives for this purpose [28]. However, the use of polyaromatic synthetic units raised concerns in terms of their fate *in vivo* and potential toxicity arising from biological applications of such materials [29-30]. For this reason, there is a very active search towards self-assembling ultrashort peptides that do not contain such capping groups. However, this task is very challenging due to the high inherent flexibility of peptide molecules, as exemplified by the finding of only four new hydrogelators from the *in silico* screening of all 8,000 combinations of L-amino acids in tripeptides [20].

Alternatively, the combination of D- and L-amino acids proved to be a successful strategy for the design of hydrophobic, self-assembling, unprotected tripeptides [31-34]. Net segregation of hydrophilic and hydrophobic components is key to achieve an amphiphilic supramolecular organization that renders the hydrogels stable [31]. Only

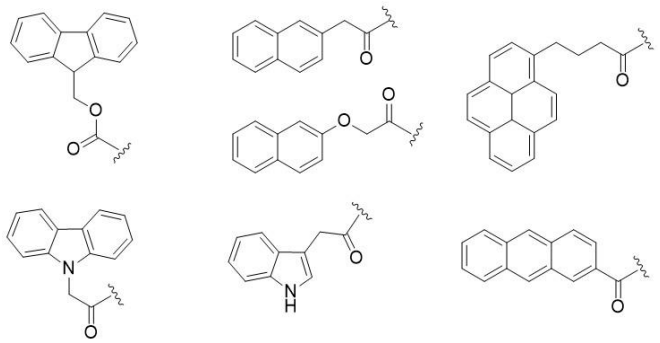


Fig. 3. N-capping groups successfully used in the design of ultrashort peptide hydrogelators.

recently, the divergent path of L-homochiral and D,L-heterochiral tripeptides towards self-assembly was monitored *as a continuum* from single molecules to macroscopic hydrogels, highlighting key differences between stereoisomers [33]. Heterochiral hydrogelators offer a number of advantages over their homochiral counterparts [35]. In particular, an attractive feature of these systems is the possibility to fine-tune their protease-mediated degradation rate; this does not only depend on the number and position of D-amino acids along the sequence, but also on the level of supramolecular packing that can effectively mask sensitive peptide bonds from hydrolytic enzymes [36]. These hydrogels are also reversible, so that simple triggers such as variations of temperature or pH could be used to switch back and forth between assembled and disassembled states, as shown for instance in Fig. 2 [32, 33].

2.2 Amyloid peptides for self-assembly

As described above, diphenylalanine is one of the most popular self-assembling motifs and it was identified by Reches and Gazit through a reductionist approach from the Abeta sequence [22]. The study searched for the minimalist sequence of the peptide involved in Alzheimer's disease that displayed a strong tendency towards self-assembly into amyloid fibrils [22]. Indeed, the vast majority of self-assembling ultrashort peptides have a certain amyloid character, defined in terms of supramolecular organization in the so-called cross-beta pattern [37], and with characteristic fibril nanomorphology and ability to bind amyloid markers, such as Congo Red (leading to birefringence) or Thioflavin T (leading to fluorescence) [38]. However, there is no straightforward correlation between amino acid sequence and amyloid character. In any case, the strong link between naturally-occuring amyloids and designed self-assembling materials has been long recognized [39]. Attractive amyloid features lie on the ability to recruit many units of the same protein so that even a weak biological activity can transform in a potent one through repetition and avidity [39]. Additionally, amyloids can self-replicate and evolve over time. On one hand, this property is interesting for the development of smart and dynamic materials; on the other, it relates to pathological aspects of infectivity and toxicity [40].

According to the official definition of amyloid, 36 amyloid proteins have been found in humans, of which 14 are strictly associated with systemic amyloidoses, 19 occur only locally, and 3 are found in both [41]. Traditionally, amyloids have been associated to pathological states and this fact calls for caution when the intent is to use amyloid peptides for biological applications. However, in recent times, scientists have challenged this paradigm, since an increasing number of functional, non-pathological amyloids have been found also in humans [42-44]. Understanding how functional amyloids avoid toxicity (Fig. 4) can provide useful elements for the design of amyloid-inspired bioactive materials [44].

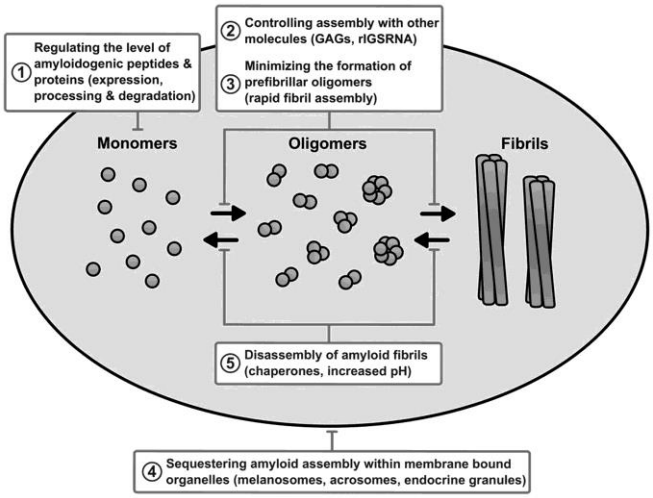


Fig. 4. Mechanisms employed in nature to avoid functional amyloid toxicity. Reproduced from ref. [44].

3. AMYLOIDS AND ANTIMICROBIALS

3.1 Functional amyloids in microbes

As described above, the amyloid state was traditionally viewed as a consequence of protein misfolding and aggregation and it is most notorious for its association with human diseases. However, a growing list of examples of "functional amyloids" challenges this bad reputation. In fact, many organisms can employ the biophysical properties of amyloid for their benefit [45]. Interestingly, microbes are amongst such organisms (Fig. 5). For instance, amyloids fulfill important microbial functions, including structure in biofilms and in the cell wall of bacterial spores, or host binding for subsequent internalization, such as the case of adhesins [46]. In addition, a recent study carried out by Seviour and co-workers established the specific binding affinity of quorum-sensing molecules to functional amyloids, which are important for cell signaling within biofilms [47]. Biofilms are communities of bacteria encased in an extracellular matrix of proteins and polysaccharides, and in many of them, amyloids are the major proteinaceous component. Within biofilms, amyloid fibers are well suited for the role of protein scaffold, they are effective on promoting adherence, and even resistance to a variety of environmental insults [48]. It is thus not surprising that inhibitors of amyloid aggregation, such as natural polyphenols, are being studied also to target biofilm formation [49].

3.2 Amyloids and host defense peptides (HDPs)

Host defense peptides (HDPs) are well-known as members of the innate immune system with antimicrobial activity. What is less known is that the activity of certain HDPs is mediated by their amyloidogenic character, which leads to the formation of amyloids to permeabilize microbial

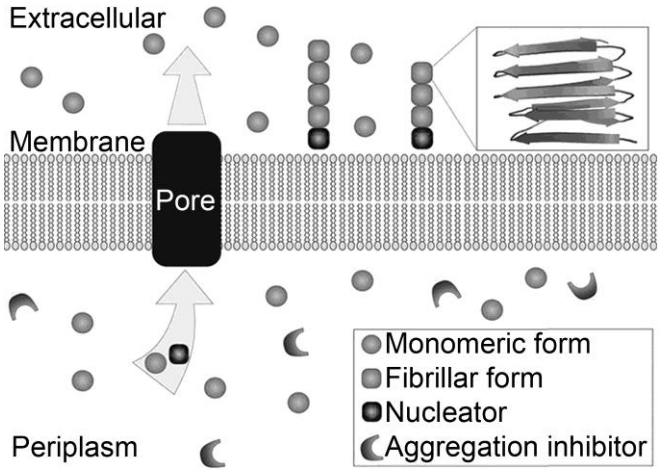
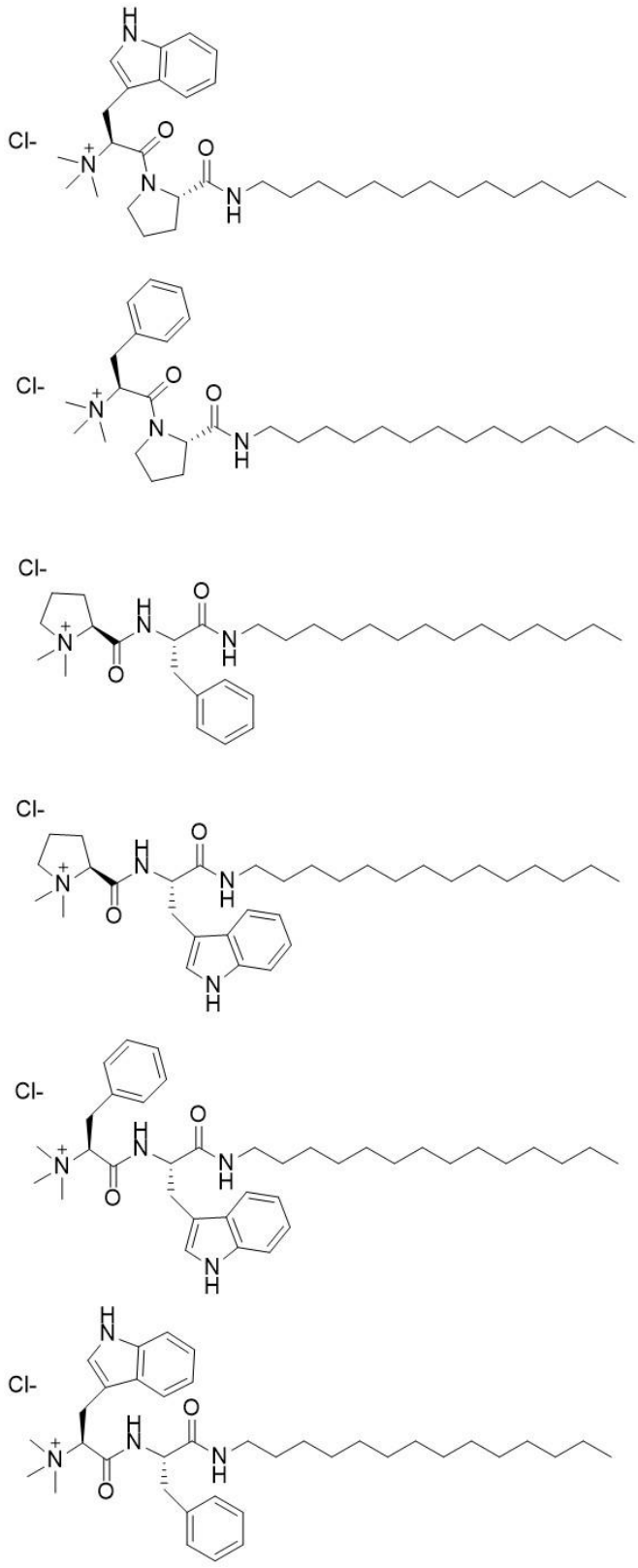


Fig. 5. Schematic mechanism of the formation of fibrils for functional bacterial amyloids *in vivo* with common components for *P. fluorescens* and *E. coli*. Adapted from ref. [50].

membranes [51]. In humans, this is the case for instance for LL-37 [52], the eosinophil cationic protein [53], lactoferrin [54], and protegrin-1 that kills microbes with a channel-forming mechanism [55]. In particular, LL-37, an HDP which – incidentally - contains the amyloidogenic diphenylalanine motif in its sequence, has been proposed to exert its antimicrobial activity in this manner, thanks to the formation of toxic oligomers [52]. In the case of the eosinophil cationic protein, the N-terminus contains a hydrophobic patch that is responsible for amyloid formation [53]. Interestingly, glycosylation was discovered as a natural means to control its toxicity and its aggregation propensity, since heavily glycosylated forms fail to form amyloids [56]. It would thus seem apparent that nature has already engineered ways to switch on/off antimicrobial activity mediated by supramolecular structures as needed, by means of post-translational modifications.

The exact mechanism by which HDP amyloids would render microbial membranes permeable is still unclear. It has been proposed that the transient state of toxic oligomers, or protofibrils, may form heterogeneous structures that intercalate in the membrane and disrupt their supramolecular organization thanks to their amphiphilic character [51]. Overall, the mechanism would seem unspecific, as found in HDPs in general [57-59]. Interestingly, it seems other mechanisms are also possible. For instance, Human alpha-Defensin-6 has been shown to form oligomers that effectively engulf microbes in a fibril net, preventing them from entering host cells [60]. Incidentally, also within the field of self-assembling short peptides used for innovative therapeutic solutions, Xu and collaborators proposed that toxic fibrils could form a net around (cancer) cells; in this case, however, cell death would result in the form of apoptosis [61]. It would thus seem plausible that not only HDPs, but also amyloids, could be a source of inspiration to



design new therapeutic solutions based on self-assembling peptides.

3.3 Amyloid Abeta as antimicrobial peptide

A recent investigation has provided data supporting an *in vivo* function for Abeta as an antimicrobial peptide. The experiments carried out reveal that Abeta exerts antimicrobial activity against eight clinically relevant microorganisms with a potency analogous to, or in some cases even higher than, LL-37, an archetypal human antimicrobial peptide [62]. Besides bacteria and fungi [62, 63], also viruses can be effectively targeted by Abeta peptides [64]. These findings, together with the observation that Abeta is highly conserved across vertebrates [65] suggest that it may act as important and natural defense against microbial

infections [66]. Evidence in this direction has been recently reviewed [67]. As a result of these investigations, the traditional "amyloid cascade hypothesis", which sees exclusively a pathological role for Abeta, has been challenged with new theories that call for a role for infections in Alzheimer's disease, and possibly a dysregulation of the innate immune response [68].

4. DESIGNER ULTRASHORT PEPTIDES AS SUPRAMOLECULAR ANTIMICROBIALS

Self-assembling ultrashort peptides designed to form nanoarchitectures and even (nanostructured) macroscopic hydrogels hold great potential as novel antimicrobial materials [4]. They can also be envisaged as vehicles to work in synergy with antibiotics or antibacterial agents [69], including nanoparticles [70]. The latter can also be formed *in situ* by including a metal-coordinating motif in the peptide sequence, thus yielding an antibacterial metallogel [70]. We can distinguish different chemical classes of peptide-based gelators, of which numerous examples have been described in the literature [26]. However, if we restrict the focus to small molecules featuring up to three amino acids, with assembling ability and antimicrobial properties, then the list of examples reduces drastically. Recently, also an N-protected amino acid (*i.e.*, *N*-(4-nitrobenzoyl)-Phe) was reported to self-assemble into a mildly antimicrobial hydrogel [71]. More generally, we can distinguish lipopeptides, Fmoc-amino acids and Fmoc-peptides, and unprotected peptides, as described below.

4.1. Lipopeptides

Das and collaborators reported a series of gelling cationic dipeptide amphiphiles (Fig. 6) that showed remarkably low minimum inhibitory concentrations (MICs) in solution against the growth of both *Gram positive* and *Gram negative* bacteria. Despite the cationic character, no cytotoxicity was observed against mammalian cells *in vitro* [72].

More recently, diphenylalanine conjugated at the N-terminus with a long aliphatic chain was reported to effectively self-assemble into a hydrogel at physiological conditions and display antimicrobial activity against *Gram negative* strains,

Fig. 6. Gelling cationic dipeptide amphiphils with antibacterial activity [72].

namely *E. coli* and *P.aeruginosa* [73]. Interestingly, substitution of one phenylalanine with phenylglycine

hindered gelation and had a negative impact on the antimicrobial activity observed [73].

4.2 Fmoc-peptides

Fmoc-Phe displayed antibacterial activity against *Gram positive* bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), both in solution and hydrogel phase [74]. The bactericidal activity was noted also at concentrations equal to, or above, the critical micelle concentration, while bacterial growth was inhibited also at lower amounts [74]. It was inferred that the hydrogel exerted activity mainly through the release of Fmoc-Phe in solution, which acted as a surfactant [74].

Das and co-workers have also demonstrated that cationic derivatives of Fmoc-Phe, Fmoc-Phe-Phe, and similar analogues, modified at the C-terminus with a pyridinium moiety, displayed antibacterial activity in solution against *Gram positive* and *Gram negative* strains [75]. At higher concentrations, these compounds were also capable of self-organization into supramolecular hydrogels, giving scope for their use as bioactive soft materials [75].

Fmoc-Phe could also co-assemble with Fmoc-Leu to form a *Gram-positive* selective bactericidal hydrogel that did not elicit mammalian cell cytotoxicity, giving scope for its use as active coating for sanitation in clinical settings [76]. Fluorination of the benzene unit of Fmoc-Phe to the pentafluoro-derivative also proved to be an effective strategy to obtain antibacterial composites when combined with a resin material [77].

4.3 Unprotected peptides

Despite the fact that the discovery of diphenylalanine self-assembly dates back to 2003, only in 2017 it was shown to be able to disrupt bacteria cell membranes in its self-assembled form [78]. Unfortunately, the fibrils did not yield stable hydrogels on their own [23, 24], but can be incorporated into hydrogels to confer them with antibacterial properties [78].

Simple addition of a third amino acid to yield a D,L-heterochiral sequence is sufficient to obtain a supramolecular hydrogel at physiological conditions [79]. This hydrogel demonstrated mild antibacterial activity against *Gram negative* strains, including a clinical isolate [80]. Importantly, this activity could synergize with that of antibiotics, if they participate in the supramolecular organization of the hydrogel for sustained release [80]. This approach could be highly attractive for the prolonged delivery of poorly soluble drugs, and could be extended to anti-inflammatory molecules [81] for a combined therapy, or even to fluorescent dyes for theranostics [82]. Unfortunately, achieving co-assembly between this hydrogelator and drug molecules is far from trivial, and rather difficult to predict based solely on chemical structures [83]. Indeed, the ability of multiple small molecules to participate together in the formation of co-assembled or self-sorted supramolecular hydrogels is a research area that has attracted wide interest amongst chemists [84-89].

CONCLUSION

The research areas of antimicrobial peptides, pathological and functional amyloids, and self-assembled hydrogels are fast-moving fields that in recent years have shown to have a lot in common. Therefore, knowledge transfer from one or even two of these fields onto the other has the potential to accelerate progress towards the development of smart supramolecular systems for innovative therapeutic solutions. Considering the worldwide emergency we are all facing in terms of spreading of antimicrobial resistance, we cannot afford to miss the research advancement opportunities that lie at the interface between these areas, and between the disciplines of medicinal, biological, organic and supramolecular chemistry, together with nanotechnology and materials science.

A key aspect that is often underlooked is the importance of an effective delivery agent capable to maximize AMP and/or drugs residency at the desired target site [90]. Recent advancements in this field have shown that peptide-based nanostructures could indeed serve this purpose leading to increased drug efficacy [91-96]. For instance, a tripeptide was recently reported to self-organize into coatings that could load and release drugs to render surfaces anti-fouling and antimicrobial [97]. Conjugation of an antimicrobial peptide to a self-assembling amphiphile was a successful strategy to eradicate biofilms formed by *P. aeruginosa* and *C. albicans* [98]. However, self-assembly was also reported to have a negative effect on the correct release of an anti-viral peptide, highlighting the challenges of this field [99]. The holy grail is now the development of smart systems that acquire potent antibacterial activity through self-assembly only when and where needed, to then disassemble into inactive and biodegradable components that are innocuous for the environment and the ecosystems.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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