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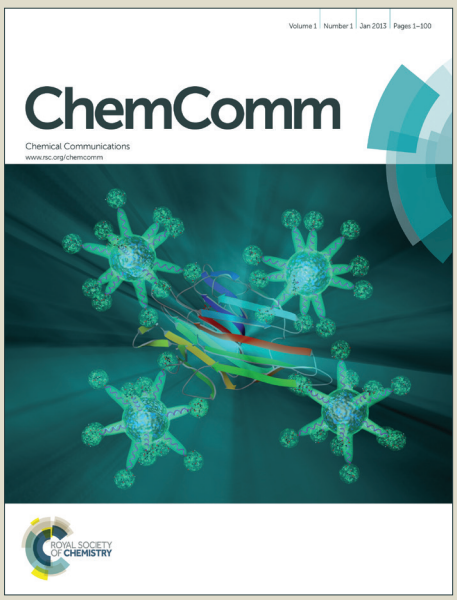

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Antimicrobial peptide shows enhanced activity and reduced toxicity upon grafting to chitosan polymers

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Here we report that grafting of a short antimicrobial peptide, anoplin, to chitosan polymers is a strategy for abolishing the hemolytic propensity, and at the same time increasing the activity of the parent peptide. Anoplin-chitosan conjugates were synthesized by CuAAC reaction of multiple peptides through 2-azidoacetyl groups on chitosan.

Antimicrobial peptides (AMPs) or host defense peptides are found widely in nature, where they form part of the innate immune response against bacterial infection. While there are different types of AMPs, the linear cationic α-helical are the most prominent.[1] These typically consist of short sequences of <30 amino acids with a high degree of cationic residues, mainly lysines and arginines, and with amphipathic character. Many AMPs attain their function by interacting with the cell membrane of bacteria. Several mechanisms for the interaction of AMPs with bacterial membranes have been proposed, however, in all of them multiple copies of antimicrobial peptides act together on the membrane.[1b]

AMPs hold great promise as potential biopharmaceutical drugs for the treatment of infections. However, clinical applications of AMPs have to overcome a number of significant challenges, including (i) the toxicity, due to the relative high minimum inhibitory concentration, (ii) the low selectivity for bacterial membranes, and (iii) their short half-life *in vivo*.[2] Many natural AMPs, e.g. melittin (causing 50% hemolysis at 8 μg/mL),[3] or synthetic AMPs, e.g. $(Arg\text{-}Trp)_5$ (causing 50% hemolysis at 76 μM)[4] exhibit excellent antimicrobial properties, however, these candidates are highly toxic towards red blood cells. This has led to a search for AMPs with high hemocompatibility.

Anoplin, a decapeptide isolated originally from the venom of the solitary wasp,[5] is an example of an AMP that displays a relatively low hemolytic activity. It has a heptad repeat sequence and is one of the shortest naturally occurring AMP structures known. Efforts to enhance its antimicrobial potency by rational sequence modifications have proven successful, however, this often lead to a concomitant, dramatic increase in hemolytic activity.[6]

These challenges have generated an interest in studying possible cluster effects in the multivalent display of AMPs. This has been the subject of several comprehensive reviews.[2,7] Interesting examples include linking of AMPs, including anoplin,[8] to scaffolds,[9] dendritic structures,[10] and linear or branched polymers.[11] While multivalent display of AMPs in some cases have proven effective,[8,12] in other cases it was ineffective.[13]

We envisioned that an improvement in antimicrobial activity could be obtained by coupling of multiple copies of anoplin monomers to a linear, biocompatible carbohydrate polymer, chitosan, for multivalent display. Here we report our initial findings in grafting anoplin peptides onto chitosan polymers, through azido moieties anchored on the 2-amino groups, by using copper-catalyzed alkyne-azide coupling (CuAAC) chemistry.[14] The conjugates consist of a chitosan backbone with anoplin peptides as branches (Fig. 1). Surprisingly, we have found that not only does this approach lead to an enhancement of antimicrobial potency, but at the same time these constructs are essentially non-hemolytic.

In order to determine the optimal orientation of anoplin monomers towards the bacterial membranes we synthesized anoplin peptides having either N- or C-terminal alkyne groups. Peptide **1** was synthesized with an N-terminal 4-pentynoic amide in lieu of the free N-terminus, whereas peptide **2** had a C-terminal, secondary *N*-propargylamide rather than the primary carboxamide of anoplin (Fig. 2).

Chitosan is a natural polymer comprised of β(1→4)-linked glucosamine and some degree of *N*-acetyl glucosamine, that has attracted special attention in recent years within a wide diversity of reported biomedical applications, also including

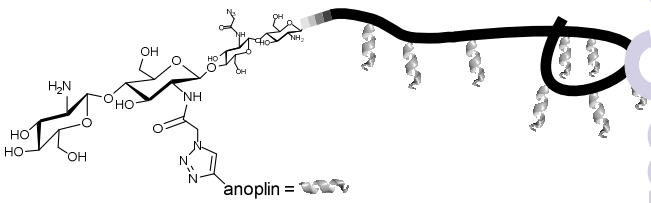


Fig. 1 Schematic representation of multivalent anoplin-chitosan conjugate. Helices: anoplin; Black ribbon: linear chitosan polymer.

antimicrobial agents.[15] In particular, chitosans quaternized at the 2-amino groups,[16] or with quarternized ammonium groups in proximity of the backbone,[16a,17] display excellent antimicrobial activities, whereas chitosan itself exhibits low antimicrobial activity only under acidic conditions when its amino groups are protonated.[3] N-Selective modification of native chitosan polymers has proven to be difficult and has led to the development of several protecting group strategies.[17a,18] One of the authors of this communication has previously introduced *tert*-butyldimethylsilyl (TBDMS) protection,[18c] for efficient masking of the 3,6-OH groups, in order to obtain completely selective N-modification. 3,6-*O*-Di-TBDMS chitosan **3** shows high solubility in common organic solvents, gives few side-reactions in subsequent substitution reactions, and can in some cases provide near-complete substitution at the amino group. This has been observed, e.g, in studies involving the introduction of various quaternary ammonium moieties,[16a] or hydrophobic substituents.[16b,19] We utilized the TBDMS technique to install 2-azidoacetyl groups on chitosan (Fig. 2). Thus, 3,6-*O*-di-TBDMS chitosan **3** was acylated with *in situ* generated 2-azidoacetic acid[20] using a carbodiimide coupling reagent. In order to vary the degree of substitution, we performed the acylation with three different ratios of acylating reagents relative to chitosan glucosamine units. Conversions amounting to 13%, 24%, and 27% were obtained by using 0.5, 0.7, and 1 equivalents, respectively, and it was not possible to acylate more than 30% of glucosamine units, even in the presence of 10 equivalents of coupling reagents under these conditions. 2-Azidoacetylation could alternatively be performed in two steps using bromoacetylbromide followed by substitution with sodium azide, however, in this case some cross-linking of chitosan was observed (data not shown). After global deprotection of the TBDMS groups under acidic conditions, 2-azidoacetyl chitosans **5** were obtained in 56-99% isolated yields. Deprotection under these conditions is known to be accompanied by trimming of the chitosan backbone, however, hydrolysis of the glycosidic bonds occurs primarily during the preparation of the TBDMS-protected precursor.[17b,21] The average degree of polymerization (DP_{Av}) of the chitosan backbone was reduced from 567 glucosamine units in the chitosan precursor (M_w 94 kDa) to 205, 199, and 175 units in derivatives **5a**, **5b**, and **5c**, respectively, see Table S5 in the ESI†. The degree of azidoacetyl substitution, DS_{Az}, obtained for derivatives **5a-c** by NMR analysis were 6%, 15%, and 24%, respectively. The presence of azido groups in **5a-c** was confirmed by IR spectroscopy, showing a characteristic absorption at 2115 cm^{-1}, due to the N_3 stretching vibration.

We initially conducted a range of experiments using sub-stoichiometric copper(I) for catalysis of the azide-alkyne cycloaddition of peptide **1** or **2** with azidoacetyl chitosans **5** in aqueous media, all of which were unsuccessful (data not shown). Only when using $CuSO_4$/sodium ascorbate in excess (10 equivalents) in phosphate buffer, pH 8.0, as described by Valverde et al.,[22] did we begin to see product formation. Upon lowering the pH to 6.0 by changing the buffer to 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-pentynoic acid (1.2 equiv.) was reacted with **5b** to provide a conversion of 95% after 1 h at room temperature. The enhanced reaction rate at pH 6.0 likely reflects increased solubility of **5** upon protonation of 2-amino groups of the chitosan backbone.

Utilizing the optimized CuAAC conditions in MES buffer we coupled peptides **1** and **2** to azidoacetyl chitosans **5a**, **5b**, and **5c**, displaying different densities of azido groups. Good to excellent incorporation of anoplin was obtained by extending the reaction time to 18 h (Table 1). Correct product formation was confirmed by NMR from the presence of the aromatic triazole peak at δ= 7.96 ppm. With peptide **1** the reaction proceeded in >95% conversion with **5a**. At the higher reacetyl densities of **5b** and **5c**, conversion was lowered slightly to 85–80%. For peptide **2** the reaction was more efficient, in all cases reaching >95% conversion with azidoacetyl chitosans **5b-c**. These coupling yields are impressive considering that the macromolecular chitosan polymer reached grafting ratios (DS_{pept}) up to 23%, i.e. in which one in four monosaccharide units were substituted with the bulky peptide. The molecular weight ($M_{w,calc}$) of the conjugates increased from ~35 kDa for **6a** and approached 80 kDa for **6c** and **7c**. In comparison, conjugation between aldehyde-functionalized anoplin and 2-aminooxyacetyl-chitosans via oxime formation provided only marginal product conversion ratios, potentially due to adverse imine formation with multiple amino groups on the macromolecular chitosan structure (see ESI†). Anoplin-chitosan conjugates **6a-c** and **7a-c** were purified by extensive dialysis to remove unreacted peptide as well as excess copper reagent. The latter was complexed with ethylenediamine tetraacetate prior to dialysis to aid removal. Trace metal analysis by ICP-MS showed that copper was efficiently removed by this method (ESI†).

With the six anoplin-chitosan conjugates at hand, we assessed the antimicrobial activity towards two Gram-positive

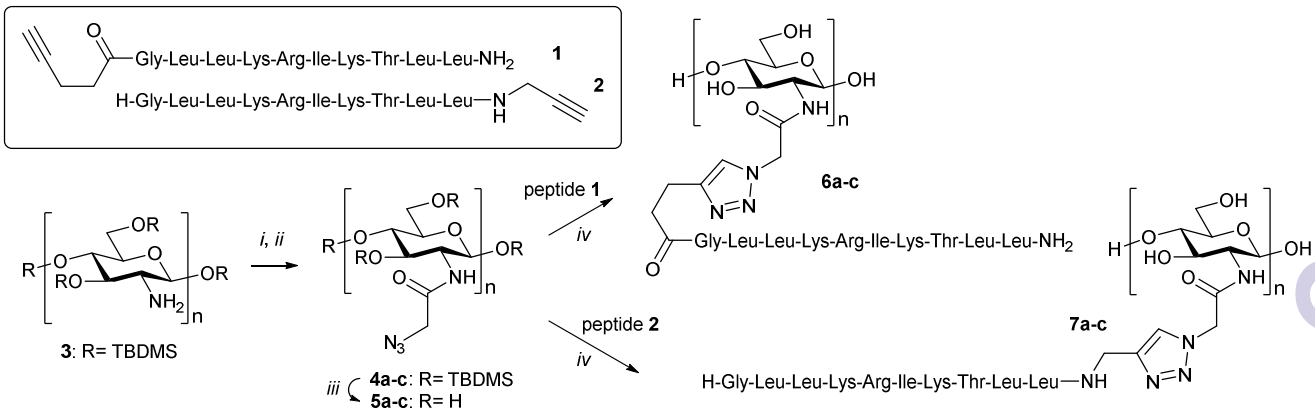


Fig. 2 Conjugation of anoplin through the N-terminus (**6**) or C-terminus (**7**) to chitosan polymer. Conditions: (*i*) Bromoacetic acid (**a**: 0.5 equiv, **b**: 0.7 equiv, **c**: 1 equiv), NaN_3, DMF, 18 h; (*ii*) **3**, EDC, HOBt, DIEA, DMF, 18 h; (*iii*) 0.8 M HCl, dioxane, MeOH, 18 h; (*iv*) Peptide **1** or **2**, $CuSO_4$, sodium ascorbate, MES buffer, pH 6.0, 18 h.

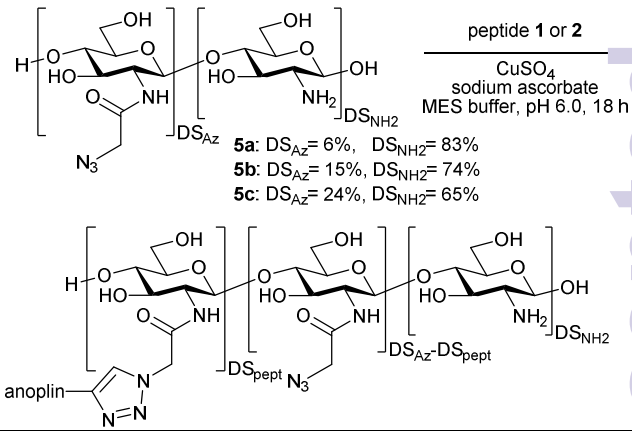
as well as two Gram-negative bacterial strains (Table 2). The conjugates generally displayed improved activities towards the four strains, as compared to anoplin. Interestingly, the broad spectrum and profile of activity of anoplin was maintained for the conjugates. The activity of the conjugates was found to be particularly significant against the Gram-negative bacteria, e.g. *E. coli*, with minimum inhibitory concentration (MIC) values as low as 4 μg/mL for **7b** and **7c**. This effect may be due to the difference in peptidoglycan density in the cell wall resulting in a lower accessibility of the polymeric conjugates towards Gram-positive bacteria. The grafting density of anoplin was found to influence antimicrobial activity in several cases, as evaluated by referencing MIC values to peptide mass (values given in parentheses in Table 2). In particular, both conjugate series **6** and **7** displayed proportional activity enhancements against *S. aureus* and *E. coli* with increasing grafting densities. In contrast, increasing grafting density led to a proportional decrease in activity against *E. faecalis* for both conjugate series. The orientation of the peptides relative to the chitosan backbone was also found to influence the activity profile of the conjugates. In particular, for *E. faecalis* and *P. aeruginosa*, conjugates having N-terminal linkage were significantly more active, whereas C-terminal linkage provided only some enhancement against *E. coli*. The MIC values in all cases remained either identical or in close range (1-2 dilutions) to the minimum lethal concentration values (see ESI†) confirming that the conjugates possessed genuine bactericidal activities.

As a preliminary toxicity study, the anoplin-chitosan conjugates were assayed against human red blood cells. A hemolytic activity (HC_{50}) of 512 μg/mL was found for anoplin. As seen in Table 2, however, all anoplin-chitosan conjugates remained completely non-hemolytic up to an initially measured maximum concentration of 2,048 μg/mL. A similar reduction in hemolytic effect has also been observed in a recent study of chitosan conjugates of achiral polylysines.[23] Further high-concentration measurements of **6b**, **7a**, and **7b** showed that the conjugates displayed very low hemolytic activities (HC_{50} values >16 mg/mL and >32 mg/mL) as compared to the parent peptide (Fig 3A). The highest selectivity towards bacterial cells over mamalian cells (HC_{50}/MIC) was observed towards *E. coli*, with the **7a-c** series exhibiting significantly higher selectivity (1,000–4,000-fold) than anoplin (8-fold). The selectivity towards *S. aureus* (50–500-fold) was better for all conjugates than that of the parent peptide displaying only a 4-fold selectivity towards this strain.

Like many other AMPs, anoplin does not display any secondary structure motif in aqueous solution; only when exposed to a hydrophobic environment, e.g. sodium dodecyl sulfate (SDS) or lipid bilayers, does it adopt an amphipathic, α-helical structure which is crucial for antimicrobial activity.[5] In order to probe the α-helicity of the anoplin-chitosan conjugate we performed circular dichroism measurements. For conjugates **7a-c**, α-helicity was clearly promoted by increasing grafting density (Fig 3B), whereas this effect was less pronounced for **6a-c** (ESI†). In all cases, the conjugate structure facilitated peptide folding even in the absence of SDS, indicative of a cooperative, intramolecular folding process for the conjugates. Collectively, these data show that anoplin maintains its ability to adopt the proper secondary fold for antibacterial activity in the conjugates. The length of the anoplin helix is known to be too short to span a lipid bilayer, and the bactericidal action has previously been ascribed to a toroidal pore mechanism.[24] It seems likely from our data that the enhancements in antibacterial activity, e.g. against *E. coli*, are promoted by the ability of the conjugates to form intramolecular, pore-forming clusters of α-helical anoplin.

In summary, we have shown that anoplin-chitosan conjugates may be synthesized by CuAAC chemistry with a high degree of control over the resulting peptide grafting density. These conjugates showed ample enhancements in

Table 1 CuAAC reactions of alkynyl peptides and 2-azidoacetyl chitosan[a]



Product	DS_{pept} (%)[b,c]	$M_{w,calc}$ (kDa)[d]	Average no. peptides/chain	Conversion (%)[c]	Yield (%)[e]
6a	6	50	12	>95	96
6b	13	67	26	85	85
6c	19	74	33	80	80
7a	6	50	12	>95	92
7b	15	71	30	>95	97
7c	23	81	40	>95	87

[a] *N*-Acetylated glucosamine units are not shown, DA= 11%. For conjugate DS values, see ESI†; [b] Peptide grafting ratio of product; [c] Determined by NMR; [d] Calculated average molecular weight, see ESI†; [e] Isolated yield.

Table 2 Antimicrobial evaluation of anoplin-chitosan conjugates towards four bacterial strains as compared to anoplin and chitosan at pH 7.2.[a]

Compound	Gram-positive bacteria *S. aureus* MIC (μg/mL)	*E. faecalis* MIC (μg/mL)	Gram-negative bacteria *E. coli* MIC (μg/mL)	*P. aeruginosa* MIC (μg/mL)	Hemolytic activity HC_{50} (μg/mL)	Selectivity $HC_{50}/MIC_{E.\ coli}$
chitosan[b]	≥ 1,024	-	≥ 1,024	-	-	-
anoplin	128	512	64	512	512	8
6a	256 (72)	128 (36)	32 (9.1)	64 (18)	≥ 4,096[c]	≥ 130[c]
6b	128 (57)	256 (113)	16 (7.1)	64 (28)	32,768	2,100
6c	64 (33)	512 (267)	16 (8.3)	256 (134)	≥ 4,096[c]	≥ 260[c]
7a	256 (73)	1,024 (293)	8 (2.3)	256 (73)	32,768	4,100
7b	128 (62)	1,024 (497)	4 (1.9)	256 (124)	16,384	4,200
7c	64 (37)	1,024 (591)	4 (2.3)	256 (148)	≥ 4,096[c]	≥ 1,000[c]

[a] MIC values for **6a-c** and **7a-c** in parentheses and selectivities are referenced to peptide mass using Table S2. For a graphical representation, see Fig. S2 in the ESI†; [b] Data from reference[3]; [c] The maximum tested concentration was 2,048 μg/mL, at which the compound was completely non-hemolytic, see ESI†

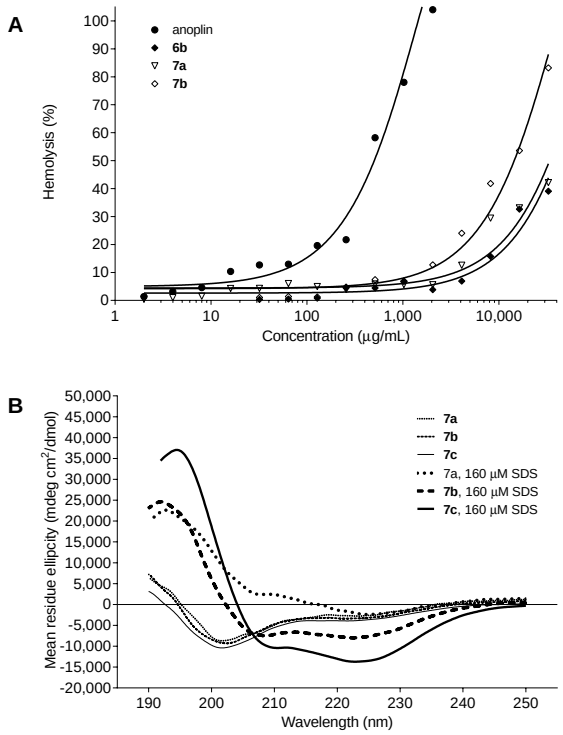


Fig. 3. (A) Hemolytic activity of anoplin and conjugates. (B) Circular dichroism spectra of conjugates **7a-c**, referenced to mean peptide residue concentration.

antibacterial activity over the parent peptide, in particular against Gram-negative bacteria, while displaying an essentially non-hemolytic behaviour. We believe that the present approach may constitute a general strategy for enhancing the activity and selectivity of AMPs. Future efforts will be directed towards optimizing the degree of polymerization of the conjugates as well as investigating the scope and mechanism of these findings by examining alternative AMP-chitosan conjugates.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis and additional characterization data. See DOI: 10.1039/c000000x/

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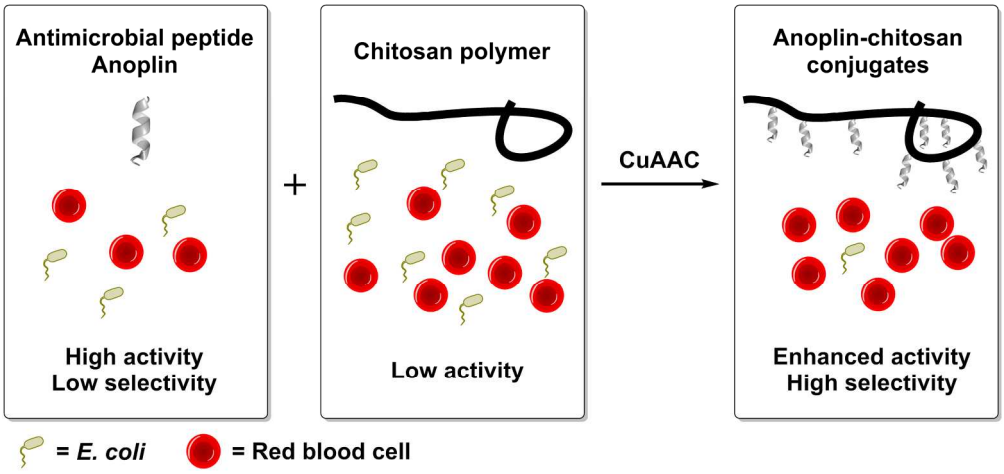

A strategy for enhancing the activity and selectivity of antimicrobial peptides (AMPs) is presented. The AMP anoplin is grafted to chitosan polymers in varying density and orientation leading to 30-fold activity enhancement and a reduction in toxicity towards red blood cell of more than two orders of magnitude.