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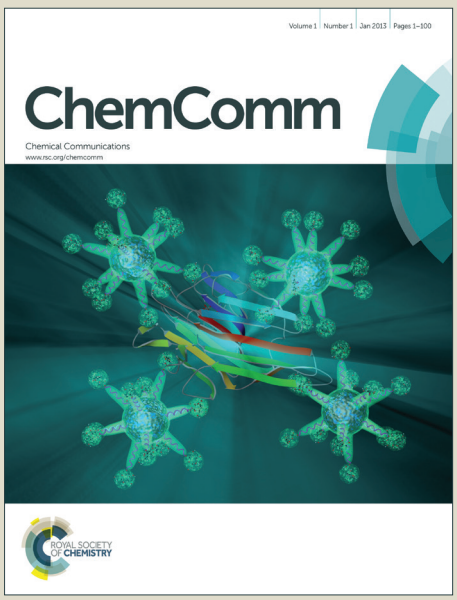



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# Amino group binding peptide aptamer with double disulphide-bridged loops selected by *in vitro* selection using cDNA display

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**Peptide aptamers that specifically bind to the amino group on a solid-phase were screened by *in vitro* selection using the cDNA display method. The identified peptides had a unique structure containing two cyclic loops with disulphide bonds and a linkage region, which were indispensable for molecular recognition.**

Recently, the molecular recognition abilities of peptides have been applied in several fields, for example in biological science,[1] material science,[2] and medical science.[3] To date, peptide binding to biomolecules, as well as inorganic surfaces (metals, semiconductors, and metal oxides) and organic molecules (nanocarbons, polymers and peptide assemblies) has been designed by *in vitro* selection manner.[4] Material-binding peptides with molecular-recognition and self-assembly capabilities are new smart materials. However, there are only a few examples in the literature of *in vitro* selection of small molecule-binding peptides; thus small molecule recognition by peptides is still an unexplored territory. In medicine, constrained peptides, such as disulphide-rich peptides including venom peptide 'conotoxins'[5] and polycyclic peptides,[6] have been investigated because of their extreme specificity and affinity towards a target protein. However, molecular recognition ability of constrained peptides containing disulphide bonds against non-protein target molecules including small molecules has not been investigated yet. Thus, we examined how a constrained peptide containing disulphide bridges can enhance the molecular recognition ability towards non-protein molecules.

cDNA display method is a technology which can covalently link a cDNA molecule and its coding protein using a puromycin-linker,[7] that is an improved version of the mRNA display method.[8] In the cDNA display method, disulphide bridges in the displayed peptide easily can form on magnetic beads in the preparation process.[9] Thus, this method is suitable to screen target molecule-binding disulphide-rich peptides from a DNA library of coding cysteine-rich peptides in which the cysteine position is randomized. In this study, an amino group on the polystyrene-based magnetic bead was chosen as the low molecular weight target, because a functional group recognizing peptides has not been reported yet.

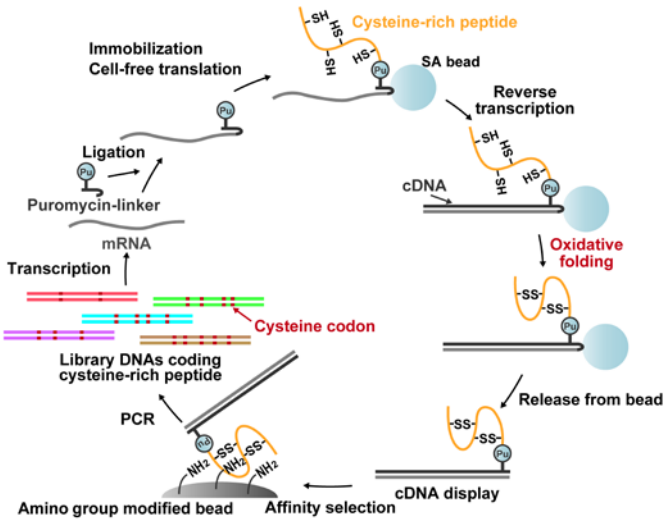


**Fig. 1** Schematic representation of the *in vitro* selection of amino group binding peptides. The initial DNA library was transcribed into an mRNA library and subsequently converted to an mRNA/cDNA-peptide fusion molecule library on a streptavidin (SA)-coated magnetic bead. Intramolecular disulphide bridges in the peptide were formed by air oxidation on the bead. The mRNA/cDNA-peptide fusion molecule library was released from the bead by RNase T1 treatment to give a cDNA display library. The cDNA display library was incubated with an amino group ($NH_2$) modified magnetic bead. The bound cDNA display molecule was collected and the DNA moieties amplified via PCR. The amplified DNA moieties were used for the DNA library in the next round of selection. After performing this selection four times, peptides binding to amino groups were enriched, and sequences were analysed via cloning and sequencing of the cloned DNA molecules. Pu = puromycin.

The *in vitro* selection process using cDNA display with a cysteine-rich peptide coding DNA library is schematically shown in Fig. 1 and described in detail in the ESI.† In brief, cDNA display molecules with disulphide-rich peptides were prepared from the cysteine-rich peptide coding DNA library. The cDNA display library was then incubated with amino-

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**Table 1 Chemically synthesized peptides.**

| No. | Name | Size (aa) | Disulphide bridge | Amino acid sequence (N→C) |
|---|---|---|---|---|
| 1 | CP1(2SS)-α | 34 | C7-C15 and C29-C32 | FAM-GGGSMGCGPSNFDDCPRNYTFNSIDSGYCSGCNH |
| 2 | CP1(2SS)-β | 34 | C7-C29 and C15-C32 | FAM-GGGSMGCGPSNFDDCPRNYTFNSIDSGYCSGCNH |
| 3 | CP1(2SS)-γ | 34 | C7-C32 and C15-C29 | FAM-GGGSMGCGPSNFDDCPRNYTFNSIDSGYCSGCNH |
| 4 | CP1(Cys→Ala) | 34 | – | FAM-GGGSMGAGPSNFDDAPRNYTFNSIDSGYASGANH |
| 5 | CP1(1SS)-A | 34 | C7-C15 | FAM-GGGSMGCGPSNFDDCPRNYTFNSIDSGYSSGSNH |
| 6 | CP1(1SS)-B | 34 | C29-C32 | FAM-GGGSMGSGPSNFDDSPRNYTFNSIDSGYCSGCNH |
| 7 | CP1(1SS)-NTR | 15 | C7-C15 | FAM-GGGSMGCGPSNFDDC |
| 8 | CP1(1SS)-CTR | 15 | C5-C8 | FAM-GGGSCSGCNH |

group modified magnetic beads. Weakly-bound cDNA display molecules were washed out and the remaining cDNA display molecules were collected by elution using sodium lauryl sulphate (SDS). The cDNA moieties of the cDNA display molecules were amplified by PCR for the next round of the *in vitro* selection. After a total of four rounds of selection were performed, the library DNA molecules were cloned and sequenced. Fifteen clones were found to have almost the same sequence among the ninety sequenced clones as shown in the ESI.†

The maximum consensus sequence peptide contained four cysteine residues and was named CP1. The peptide CP1 has three kinds of disulphide connectivity. To investigate which disulphide isomers can recognize amino groups on the bead, the three disulphide isomers of CP1 (i.e., CP1 (2SS)-α, CP1 (2SS)-β and CP1 (2SS)-γ; see Table 1) were chemically synthesized and assayed by the pull-down method using amino group modified beads. The captured peptides were detected with N-terminal modified fluorescein (FAM) after SDS-PAGE using a fluorescence image analyser (BioRad, USA). Interestingly, only peptide CP1 (2SS)-α was shown to interact with amino group modified beads, the other two disulphide isomers, CP1 (2SS)-β and CP1 (2SS)-γ, did not interact (Fig. 2). These results indicate that specific disulphide connectivity in the CP1 peptide is required for recognition of the amino group on the bead. Next, we examined whether CP1 (2SS)-α could recognize an amino group on a different solid-phase. Buffer solutions containing each disulphide isomer of CP1 were spotted onto an amino group modified slide glass (Mastunami Glasses, Japan). After the unbound peptides were washed out, the remaining peptides on the glass slide were directly imaged with a fluorescent image analyser. The results indicate that only peptide CP1 (2SS)-α was able to bind the amino group on the glass slide. The other disulphide isomers: CP1 (2SS)-β and CP1 (2SS)-γ, and the peptide CP1 (Cys→Ala) where cysteine is replaced with alanine could not bind the amino group (Fig. 3a). We also examined whether these peptides could bind amino groups on agarose-resin beads. Only CP1 (2SS)-α was able to recognize the amino groups on the agarose-resin beads under the oxidation conditions (Fig. 3b). Furthermore, the peptide CP1 (2SS)-α could bind only amino group modified agarose-resin beads, but could not bind either non-modified or carboxyl group modified agarose-resin beads (Fig. S2, ESI†). Thus, these results suggest that CP1 (2SS)-α can recognize amino groups regardless of the nature of the solid phase that the amino groups are immobilized on. We believe that this is the first example where a peptide containing disulphide bridges is shown to recognize moieties as small as amino groups.

In previous studies, many kinds of cyclic peptides containing a disulphide bond have been developed to achieve high affinity and specificity against proteins.[3c] Although CP1 (2SS)-α that was selected by *in vitro* selection contains two disulphide-bridged loops, it is uncertain whether both these constrained cyclic loops are indispensable for recognition of the amino group. Hence, to investigate whether both loops are required for recognition, all possible combinatorial pattern peptides of CP1 (2SS)-α which have only a single cyclic loop, with or without each remaining region in which cysteines were substituted to serines, were chemically synthesized (Table 1). According to a binding assay of these peptides against amino groups on magnetic beads, only CP1 (2SS)-α could recognize the amino group (Fig. 4). This indicates that both cyclic loops in the peptide CP1 (2SS)-α are required for amino group recognition. Moreover, the flexible region between the cyclic loops may also be required for molecular recognition. In a previous study, we found that a peptide with two disulphide

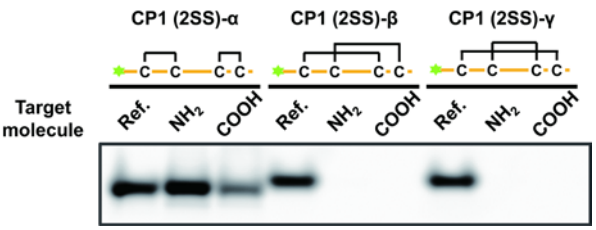


**Fig. 2** Binding assay of the disulphide isomers of CP1 (2SS): CP1 (2SS)-α, β, and γ against amino groups or carboxyl groups by a pull-down assay. Each disulphide isomer of CP1 (2SS) was incubated with amino group ($NH_2$) or carboxyl group (COOH) modified magnetic beads at 25 °C for 2 h in a selection buffer (20 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 0.1% Tween20). After washing the beads, the remaining CP1 was eluted with SDS sample buffer. The eluates were subjected to tricine-SDS-PAGE and visualized with the fluorescence image analyser. A sample (3.3%) of each imputed disulphide isomer of CP1 (2SS) was loaded as a reference (Lane: Ref.).

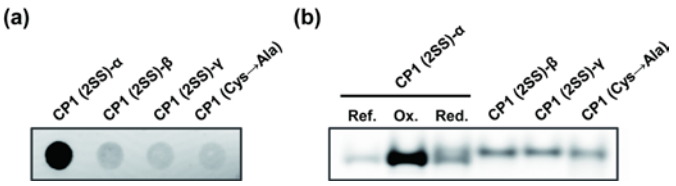


**Fig. 3** Binding assay of CP1 (2SS)-α against amino groups on several types of support. (a) The three disulphide isomers of CP1 (2SS) and CP1 (Cys→Ala) were spotted on an amino-group modified glass slide and each peptide remaining on the glass slide after washing was visualized with a fluorescence image analyser. (b) The three disulphide isomers of CP1 (2SS) and CP1 (Cys→Ala) were incubated with amino group modified agarose-resin beads. CP1-1 (2SS)-α was incubated with the agarose-resin beads under oxidizing conditions (Ox.) or reducing conditions with DTT (Red.). After washing the agarose-resin beads, each remaining peptide was eluted with SDS sample buffer. Each eluate was subjected to tricine-SDS-PAGE and visualized with the fluorescence image analyser. CP1 (2SS)-α (1 pmol) was loaded as a reference (Lane: Ref.).

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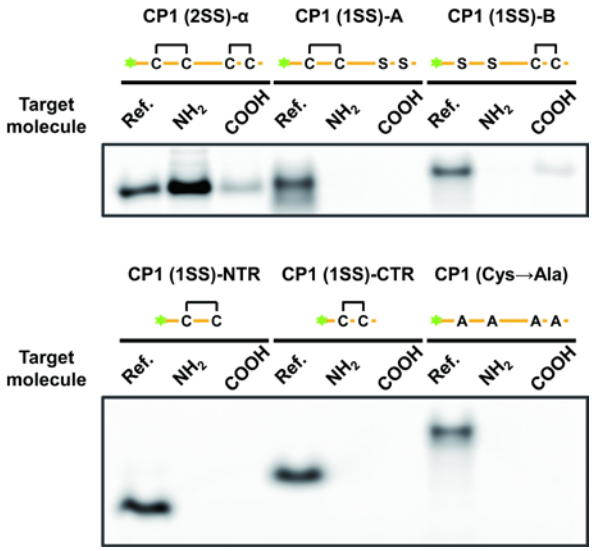


**Fig. 4** Binding assay of CP1 (2SS) derivative peptides. CP1 (2SS)-α and five derivative peptides of CP1 (2SS)-α: CP1 (1SS)-A, CP1 (1SS)-B, CP1 (1SS)-NTR, CP1 (1SS)-CTR, and CP1 (Cys→Ala) were assayed by the pull-down method under the conditions as described for Fig. 2. A sample (3.3%) of each imputed disulphide isomer of CP1 (2SS) was loaded as a reference (Lane: Ref.).

bridges interacts with the interleukin 6 receptor with high affinity and specificity.[7a] However, the disulphide connectivity in this peptide is similar to CP1 (2SS)-γ, which is different to CP1 (2SS)-α. The disulphide bridges in CP1 (2SS)-γ causes the molecule to become rigid, but CP1 (2SS)-α, with two cyclic loops, has both rigid and flexible regions. Although CP1 (2SS)-α appears not to form a specific structure, using computer simulation we found that CP1 (2SS)-α could form some energetically-stable structures (Fig. S3, ESI†). In addition, circular dichroism (CD) spectroscopy results showed that CP1 (2SS)-α contained secondary structures, although CP1 (Cys→Ala) was in a random coil state (Fig. S4, ESI†). These results suggest that the disulphide bonds might enable the peptide to form secondary structures. In a previous study on polymer-binding peptides (7-mers) that recognize poly(methyl methacrylate), it was suggested that the rigid and kinked structure formed by proline and possible hairpin conformation of the essential 4-mer sequence is the most important aspect for peptide affinity.[10] Recently, the same authors reported the screening, by *in vitro* selection using phage display, of short 12-mer peptides recognizing the simplest polycyclic aromatic hydrocarbon, naphthalene; both the phenylalanine and proline residues were shown to be essential for creating the specific affinity.[11] A rigid steric conformation for peptides may be very important for molecular recognition by improving the ability of the peptide to interact with a molecule.

We have demonstrated that 30-mer peptides containing two disulphide bridges recognizing the amino group on a solid-phase can be screened in an *in vitro* selection manner by cDNA display from a cysteine-rich peptide coding DNA library. Pull-down assays of the disulphide isomers of CP1 peptides showed that the unique disulphide bridge pattern of the CP1 (2SS)-α peptide is required for amino group recognition. Neither of the single cyclic loop regions of the CP1 (2SS)-α peptide could bind amino groups on the solid-phase. Interestingly, not only both cyclic loops, but also the linkage region between the loops is indispensable for recognition of the amino group. Therefore, the CP1 (2SS)-α peptide containing two cyclic loops with disulphide bonds forms a unique conformation that is able to bind the amino group. This study demonstrates that peptides containing disulphide bridges can recognize functional groups of organic molecules, such as amino groups. Constrained peptides containing disulphide bridges as described in this work may represent a new approach in the molecular recognition of small molecules. Peptides can be easily linked other peptides and proteins by protein engineering with or without chemical modifications. On the other hand, amino group modification can be readily introduced to many kinds of materials. Thus, amino group binding peptide aptamers may be useful tools to compose bio-nano hybrid materials, comprising proteins/peptides and amino-modified functional materials.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: Library construct, *in vitro* selection, binding assay, circular dichroism (CD), peptide structure prediction. See DOI: 10.1039/c000000x/

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