Modification of metal surfaces using TCM-derived cyclotides: An antibacterial and antibiofilm study

Pan Cao[1], Ying Yang[2], Fidelia Ijeoma Uche[2], Sarah Hart[2], Chengqing Yuan[1], Wen-Wu Li[2]

[1]School of Energy and Power Engineering, Wuhan University of Technology, China
[2]Institute for Science and Technology in Medicine, Keele University, United Kingdom

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Introduction

Traditional Chinese Medicine (TCM) has been used in China for more than a thousand years for the prevention and treatment of various diseases including infections (1). Herbal medicine is the main component of TCM, which are grouped into many categories in the treatment of diseases. For example, the heat-clearing Chinese herbs (HCCHs) are mostly cold in nature, which "can clear away heat, purge fire, dry dampness, cool blood, and relieve toxic material."(1) Many HCCHs such as *Viola yedoensis*(2), *Coptis chinensis*, *Flos Lonicerae*, *Radix Isatidis*, and *Andrographis paniculata*, have been demonstrated to be effective in the treatment of inflammatory disease and microbial infection (1). In particular, one of the active components of *V. yedoensis* – cyclic peptides (cyclotides) are very interesting and antibacterial molecules. Cyclotides are a family of plant disulfide-rich peptides arranged in a knotted pattern which is connected *via* cysteine residues with a combinatorial cyclic backbone (3, 4). These structural features bestow on the cyclotides not only a remarkable stability, but also various biological activities including antibacterial (5, 6), antifouling effects(7) and anti-HIV(8) activities. *Viola* species (Violaceae family) contain an abundance of cyclotides (5). Recently, a series of cyclotides have also been isolated and characterized by us from several traditional Chinese herbs (6, 9, 10). *V. ordorata* and *V. arvensis* (5) are herbal medicines approved by European Medicines Agency for the treatment of skin and respiratory tract disorders and other diseases.

Bacterial biofilms are associated with more than two thirds of all infections, posing a major threat to public health. Bacteria in biofilms are highly resilient and render conventional antibiotics inefficient. Preventative strategies are appealing in comparison to allowing biofilms to form; in particular, surface modification using antimicrobial peptides may reduce biofouling by creating bactericidal, bacteria-resistant, or bacteria-repelling surfaces. However, most of peptides possess a number of disadvantages, such as susceptibility to proteolytic degradation. Hence, stable peptidomimetics need to be designed and synthesized to overcome these shortcomings (11). But these will create another obstacle for the future and wide application and commercialization due to high-cost of production, technological challenges and environmental pollution. Here, naturally occurring cyclotides, possessing remarkable stability and antimicrobial activities from a traditional Chinese Medicine (TCM) - *Viola philippica* Cav., were used for the modification of metal surfaces in order to prevent biofilm formation.

Methodology

Cyclotides were extracted and purified from *V. philippica* Cav. by HPLC, and identified using mass spectrometry. Cyclotides were subsequently utilized to modify stainless steel surfaces *via* polydopamine-mediated coupling (12). The resulting cyclotide-modified surfaces were characterized by Fourier transform infrared (FTIR) spectroscopy and contact angle analysis. The antibacterial capacity of these cyclotides against *Staphylococcus aureus* was assessed by Alamar blue assay. The antibiofilm capacity of the modified surfaces was assessed by crystal violet assay, and scanning electron microscopy (SEM) (6).

Results

A composite of Varv A, Kalata b1, Viba 15 and Viba 17 (P1), Varv E (P2), and Viphi G (P3) was isolated and identified (Table 1) . FTIR analysis of the modified surfaces demonstrated that cyclotides bound to the surfaces and induced reduction of contact angles. Antimicrobial effects showed an order P3 > P1 and P2, with P3-treated surfaces demonstrating the strongest antibiofilm capacity (Figure 1). SEM confirmed reduced biofilm formation for P3-treated surfaces (6).

Table 1: Cyclotides isolated and identified from V. philippica.

Code	Cyclotide	Sequence of amino acid residues	Theoretical monoisotopic mass	Experimental monoisotopic mass	GRAVY for their linear forms	Net charges
P1	Varv A	Cyclo-(CGETCVGGTCNTPG---CSCSWPVCTRNGLPV)	2876.17	2876.06	0.148	0
	Kalata b1	Cyclo-(CGETCVGGTCNTPG---CTCSWPVCTRNGLPV)	2890.14	2890.11	0.152	0
	Viba 15	Cyclo-(CGETCVGGTCNTPG---CACSWPVCTRNGLPV)	2860.18	2860.12	0.238	0
	Viba 17	Cyclo-(CGETCVGGTCNTPG---CGCSWPVCTRNGLPV)	2846.02	2846.08	0.162	0
P2	Varv E	Cyclo-(CGETCVGGTCNTPG---CSCSWPVCTRNGLPI)	2890.14	2890.00	0.159	0
P3	Viphi G	Cyclo-(CGESCVF I P C I SAIIGCSCSNKVCYKNGSIP)	3170.43	3170.43	0.726	+1

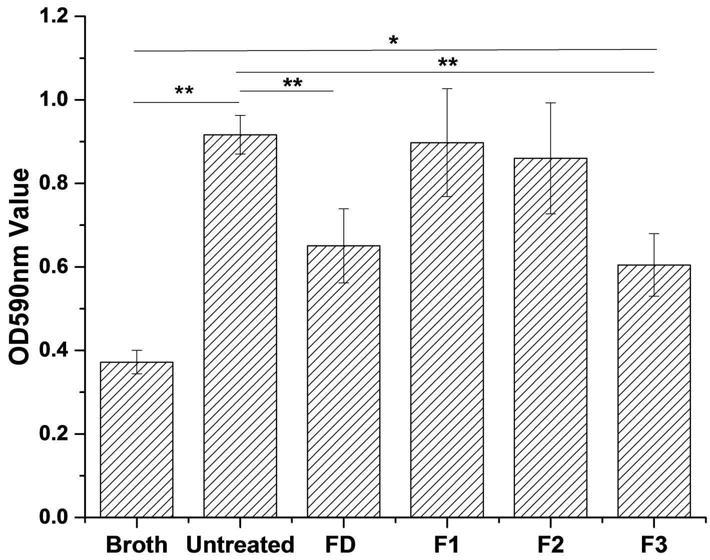

Figure 1: Comparision of antibiofilm ability of untreated, dopamine (DA)- and cyclotide-treated metal surfaces. Metal disk samples treated with DA, P1, P2 and P3 were denoted as FD, F1, F2 and F3, respectively. Optical density of the eluted acetic acid at 595 nm from the crystal violet stained metal samples after incubation with S. aureus is shown here. ANOVA was used to compare groups (, $p < 0.05$; **, $p<0.01$, $n = 4$). Error bars indicate standard deviation.*

Conclusions

In summary, cyclotides were isolated and identified from *V. philippica*. These peptides were successfully utilized to modify the surfaces of stainless steel *via* a simple, versatile and facile coupling agent, dopamine, for the first time. We therefore propose that TCM-derived cyclotides, with their unique three-dimensional structure and remarkable stability, can serve as a novel source of biological materials to be used for the modification of metal surfaces in medical devices, aquaculture, food manufacture and shipbuilding devices. This study provides novel evidence for cyclotides as a new class for development of antibacterial and antibiofilm agents (6).

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