Immmobilisation of bifenthrin for termite control

Yan-Qing Guan, a† Jia Mei Chen, a† Zhi Bin Li, a Qi Li Feng a and Jun-Ming Liub,c,d

Abstract

BACKGROUND: Termites are worldwide pests causing considerable damage to agriculture, forestry and buildings. While various approaches have been tried to eliminate termite populations, the relevant toxicants are associated with certain risks to the environment and human health.

RESULTS: In this study, to combine the merits of effective chemical control by bifenthrin and a drug photoimmobilisation technique, silk fibroin was used as a carrier to embed bifenthrin, which was then photoactively immobilised by ultraviolet treatment on the surface of wood (cellulose). The immobilised bifenthrin embedded in the photoactive silk fibroin was characterised by Fourier transform infrared spectroscopy (FTIR), ultraviolet absorption spectroscopy (UV), fluorescence measurement and CHN analysis. The surface structures and biological activity were examined by scanning electron microscopy (SEM), atomic force microscopy (AFM), electron spectroscopy for chemical analysis (ESCA) and bioassays respectively.

CONCLUSIONS: The results indicate that the embedded and immobilised bifenthrin has been very well protected from free release and has a long-term stability allowing slow release with a high efficiency against termites at a low dose of 1.25 µg cm−2. This study provides a novel and environmentally benign technique for termite control by photoimmobilising silk-fibroin-embedded bifenthrin on the surface of materials that are otherwise easily attacked by termites.

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Keywords: bifenthrin; silk fibroin; immobilise; cellulose; termite

1 INTRODUCTION

Termites are a group of insects with more than 2500 species, of which 300 species are considered as pests. Termites are one class of the most damaging pests in the tropics and can cause considerable problems in agriculture, forestry and housing. The estimated cost of damage by Formosan termites is $1 billion annually. 1 In spite of the many approaches that have been tried to eliminate termite populations, chemical control techniques are the major choices. However, the toxicants associated with these techniques offer certain risks and shortcomings. As certain soil insecticides are banned, public demand for reduced-risk, environmentally friendly alternatives has increased. 2

New chemical techniques for termite population control have increased. 2,3 Bifenthrin, a non-alpha-cyano pyrethroid, has been used against a broad range of agricultural pests and is recommended as an effective insecticide for net treatment. 3,4 Pullulan, gelatin, 5−6 the fusion protein of hepatocyte growth factor, 7 an autoantigen microarray, 8 gold particles, histidine polymer, 9 poly(acrylic acid)-on-polyallylamine microgel, 10 chargeable polymers 11 and poly(vinyl alcohol) 12 onto the surfaces of different materials for activity research in vitro. In an earlier work, the present authors reported that the coimmobilised IFN-γ and TNF-α can transduce an apoptosis signal for a longer time than soluble IFN-γ and TNF-α in human cervical cancer cells. 13 More recently, photoimmobilisation was also used to create a thermosensitive and cytocompatible AzPhPIA-coated PSt surface which was investigated by corneal epithelial cell culture. The results revealed that the AzPhPIA-coated PSt exhibits good cytocompatibility and cell detachability when temperature decreases. 14

Polymeric drugs, with advantages of delayed release and long action, have been the trend in novel drug development. For example, photoimmobilisation can be used to immobilise various peptides, enzymes, growth factors and proteins on the polymer surface in an effort to produce biomaterials with different properties and utilisations. Along this line, Ito, Chen and coworkers successfully immobilised a growth factor, 9 erythropoietin, 10 pullulan, 11 gelatin, 12 the fusion protein of hepatocyte growth factor, 13 an autoantigen microarray, 14 gold particles, 15 histidine polymer, 16 poly(acrylic acid)-on-polyallylamine microgel, 17 chargeable polymers 18 and poly(vinyl alcohol) 19 onto the surfaces of different materials for activity research in vitro. In an earlier work, the present authors reported that the coimmobilised IFN-γ and TNF-α can transduce an apoptosis signal for a longer time than soluble IFN-γ and TNF-α in human cervical cancer cells. 20

More recently, photoimmobilisation was also used to create a thermosensitive and cytocompatible AzPhPIA-coated PSt surface which was investigated by corneal epithelial cell culture. The results revealed that the AzPhPIA-coated PSt exhibits good cytocompatibility and cell detachability when temperature decreases. 21

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These earlier works set up a basis for developing novel and efficient polymeric drugs. In order to improve the performance of conventional approaches for termite control, this study was designed to examine a polymeric bifenthrin drug. A primary motivation was to explore silk fibroin as a vehicle for bifenthrin and to immobilise it on the surface of wood. Consequently, the activity of the immobilised bifenthrin embedded in silk fibroin could be inspected in animal experiments. It was clearly shown that the immobilised bifenthrin has long-term effects and almost zero release rate, by which the relevant environmental pollution problems can be remarkably reduced. More interesting is that the immobilised bifenthrin has even better performance at a lower dose $1.25 \mu g m^{-2}$. The present study thus shows a substantial potential of this novel drug to reduce termite attack in a number of practical applications.

## 2 MATERIALS AND METHODS

### 2.1 Raw materials

In preparation and testing of the polymeric materials, *Coptotermes formosanus* Shiraki was provided by HeYi Trading Co. Ltd (Guangzhou, China), and bifenthrin was purchased from Sigma-Aldrich Trading Co. Ltd (Shanghai, China). The silk fibroin used as a drug vehicle, dimethylformamide (DMF) as solvent and N-(4-azidobenzoyloxy) succinimide as crosslinking agent were purchased from Sigma-Aldrich Trading Co. Ltd (Shanghai, China) and used without further purification.

### 2.2 Synthesis and characterisation of photoactive silk fibroin

The immobilisation procedure is shown in Figs 1, 2 and 3. The preparation of photoactive silk fibroin was described previously. Briefly, silk fibroin (50 µg) was added to DMF/PBS (pH 7.4) (4:1.5 mL) solution with N-(4-azidobenzoyloxy) succinimide and mixed by stirring on ice, followed by reaction at 4 °C for 48 h. After the full reaction, the silk fibroin derivative was purified by centrifugation on membrane (Milipore Molecut II, 5 kDa) at 4000 rpm for 30 min.

The photoactive silk fibroin obtained was characterised by Fourier transform infrared spectroscopy (FTIR) (TENSOR27; Bruker, Germany; wave number range 0–4000 cm$^{-1}$), ultraviolet absorption spectroscopy (UV) (UV2450; Shimadzu, Japan; wavelength range 190–350 nm) and fluorescence measurement (Perkin Elmer LS55; Perkin Elmer, USA; wavelength range 372–530 nm and excitation wavelength 341 nm). The content of silk fibroin, azidophenyl-silk fibroin or N-(4-azidobenzoyloxy) succinimide for FTIR was 5 mg. The concentration of silk fibroin, azidophenyl-silk fibroin or N-(4-azidobenzoyloxy) succinimide for the UV and fluorescence measurement was 0.01% wt/wt. The chemical composition of the derivatives was further examined using a CHN analyser (Elementar Vario EL; Elementar Analysensysteme, Germany). The photoimmobilisation of bifenthrin was then performed.

### 2.3 Photoimmobilisation of bifenthrin and AzPhSF-bifenthrin-immobilised cellulose surface characterisation

Bifenthrin, and then the photoactive silk fibroin, were cast on raw wood chips (1 cm$^2$) and aired at room temperature. Subsequently,
Figure 4. Termite feeding experiments induced by control without bifenthrin (A and B) and 1.25 μg cm$^{-2}$ bifenthrin-coated (C and D) and 1.25 μg cm$^{-2}$ bifenthrin-immobilised (E and F) cellulose chips. A, C, E: integral graph; B, D, F: local graph.

The wood chip was UV irradiated using a UV lamp (120 W) from a distance of 5 cm for 10 s, followed by repeated washing using distilled water. The as-prepared product was azidophenyl silk fibroin (AzPhSF)-bifenthrin-immobilised cellulose (immobilised wood chip).

The surface morphology and structure of the AzPhSF-bifenthrin-immobilised cellulose were investigated by scanning electron microscopy (SEM) (JSM-6330F, Japan) and atomic force microscopy (AFM) (tapping mode, Autoprobe CP Research, CP0519; Thermo Microscopes, USA). For SEM characterisation, the samples were desiccated and gold sprayed, and then the surface morphologies of cellulose, AzPhSF-immobilised cellulose and AzPhSF-bifenthrin-immobilised cellulose were observed. For AFM probing, the samples were desiccated and fixed, and the morphology and size distribution of the AzPhSF-immobilised cellulose and AzPhSF-bifenthrin-immobilised cellulose were observed.

In addition, the immobilised cellulose was characterised by electron spectroscopy for chemical analysis (ESCA LAB 250; Thermo Fisher Scientific) equipped with Al K$_\alpha$ at 1486.6 eV and 150 W power at the anode. A survey scan and C-1s core-level scan spectra were performed, and the probed peak height offered the atomic sensitivity.

2.4 Pest experiment
For application purposes, pest experiments were performed using the as-prepared bifenthrin-coated and bifenthrin-immobilised cellulose chips for termite control. Control cellulose chips without bifenthrin, as shown in Fig. 4, were used for comparison. The termites were fed using water and wood chips with bifenthrin at different doses (1.25, 2.5 and 7.5 μg cm$^{-2}$) on dark glass petri dishes for 1–8 days. The bottoms of these dishes were loaded with sterilised sand. The death of termites was checked each day. During that period, the viable termites were counted, and the mortality of termites was quantified by the ratio of dead termites to the total number of termites. The experiment was done in three cycles, and averaged data with uncertainties are presented.

2.5 Release kinetics of bifenthrin in vitro
Given the immobilised cellulose, it was necessary to measure the release kinetics of bifenthrin in the as-prepared immobilised wood chips, which is essential for environmental evaluation. To proceed, the authors took bifenthrin-coated wood chips without photoimmobilisation (free group) and bifenthrin-immobilised wood chips for comparison study. For each target dose (1.25 μg cm$^{-2}$), the two types of wood chip were checked for release kinetics in vitro ($n = 3$). To evaluate the release effect, the wood chips were immersed in 1.5 mL of rainwater (pH 5.8) at room temperature. At preset time points (0.5, 1, 2, 3, 4, 5, 6, 7 and 24 h), the rainwater was separated from the chips, and 0.75 mL of petroleum ether was added (rainwater: petroleum ether = 2:1 by volume) to extract and concentrate the bifenthrin from the water phase. The samples were then analysed by gas-phase chromatography (GC-2010; Shimadzu, Japan; toluene solvent) to obtain the release rate relative to a treatment of 1.25 μg cm$^{-2}$ for bifenthrin-coated or bifenthrin-immobilised wood chips.

2.6 Statistical analysis
Statistical results were obtained using SPSS software. The statistical significance $P$-values were calculated using the paired Student’s $t$-test; $P < 0.05$ was considered statistically significant.

3 RESULTS
3.1 Functional group analysis of photoactive silk fibroin
A schematic diagram of substrate preparation and termite feeding is given in Fig. 1, and the new molecular structure as formed by the photoactive AzPhSF is shown in Fig. 2, as previously described. Clearly, the composition of the functional groups and the molecular structure of silk fibroin changed on photoimmobilisation, as evidenced below.

In fact, a number of amino (peak at about 3600 cm$^{-1}$) and carboxy groups exist in the silk fibroin molecule. These can be assessed by FTIR probing. For example, the azidophenyl group contained
in N-(4-azidobenzoxyloxy) succinimide peaks at 2110–2139 cm\(^{-1}\), as shown in Fig. 5. Under appropriate conditions, the carboxylic group in N-(4-azidobenzoxyloxy) succinimide and the amino group in silk fibroin can be polymerised, resulting in the formation of an amide bond and subsequent stereorecture change, which was displayed as an absorption peak at 2122 cm\(^{-1}\) (\(-\text{N}_3\)) for photoactive silk fibroin, as compared with raw silk fibroin.

Furthermore, an UV spectrum of AzPhSF is shown in Fig. 6 to illustrate the polymerisation. For the photoactive silk fibroin, the diffusive absorption at 272 nm is assigned to the azidophenyl group, while the absorption of N-(4-azidobenzoxyloxy) succinimide has a broad plateau. This difference may be due to the electron delocalisation of the azidophenyl group caused by amide bond formation. The fluorescence spectrum of the photoactive silk fibroin is shown in Fig. 7. The fluorescence profile of AzPhSF essentially remains the same as that of raw silk fibroin, although some difference in magnitude can be seen. The peak position is remarkably different from that of N-(4-azidobenzoxyloxy) succinimide, indicating that the azidophenyl groups are probably inside the silk fibroin.

The elemental analysis data for raw silk fibroin and AzPhSF are summarised in Table 1. The nitrogen content of AzPhSF increased to 24.5% from 15.8% for raw silk fibroin after reaction with N-(4-azidobenzoxyloxy) succinimide.

The above spectroscopic evidence clearly shows the effective polymerisation of silk fibroin with N-(4-azidobenzoxyloxy) succinimide, forming photoactive AzPhSF.

### Table 1. Elemental analysis of silk fibroin derivatives (AzPhSF)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C</th>
<th>H</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>38.8</td>
<td>7.4</td>
<td>15.8</td>
</tr>
<tr>
<td>AzPhSF</td>
<td>33.8</td>
<td>6.8</td>
<td>24.5</td>
</tr>
</tbody>
</table>

#### 3.2 Microscopic surface analysis of embedded and immobilised bifenthrin

After the photoimmobilisation procedure (Fig. 3), it is critical to check the attachment of the photoactive AzPhSF to the wood chips. Figure 8 presents three SEM images: for the cellulose surface (A); for the photoimmobilised silk fibroin surface (B); for the photoimmobilised silk fibroin-bifenthrin surface (C). The images reveal that the cellulose surface (A) exhibits a somewhat porous network structure. However, the photoimmobilised silk fibroin surface (B) and the photoimmobilised silk fibroin-bifenthrin surface (C) show a nanoscale network structure, which may arise from the photoimmobilisation of the silk fibroin or silk fibroin-bifenthrin do cover the cellulose surface.

AFM was further employed to probe the surface geometry, the peak height within the frame and the average roughness \(R_a\) of silk fibroin and silk fibroin-bifenthrin immobilised on the wood chip surface, as shown in Fig. 9. The peak height of the silk fibroin within the frame is 230.80 nm (A) and the \(R_a\) is 57.37 nm (C). However, the peak height of the silk fibroin-bifenthrin within the frame is 324.70 nm (B) and the \(R_a\) is 98.50 nm (D). The formation of nanoscale silk fibroin-bifenthrin on the surface suggests that, in the photoimmobilised region, the photoactive silk fibroin was crosslinked with itself, bifenthrin and substrate surface molecules simultaneously.

More direct evidence comes from ESCA survey scanning of cellulose, AzPhSF-cellulose and AzPhSF-bifenthrin-cellulose to show the chemical structure of the surfaces. The cellulose substrate showed two peaks corresponding to the C-1s (binding energy 285 eV) and O-1s (binding energy 532 eV) respectively, as expected. However, the AzPhSF-cellulose substrate showed three peaks corresponding to the C-1s, O-1s and N-1s (binding energy 400 eV). The chemical compositions of the modified cellulosics from the ESCA survey scan spectra are shown in Table 2. The O-1s content (35.8%) of cellulose decreased to 31.1% after AzPhSF immobilising. Furthermore, the N-1s component changed from 13.6 to 1.6% after the AzPhSF and AzPhSF-bifenthrin immobilisations, demonstrating the successful immobilisation of bifenthrin on the substrate.

#### 3.3 Biological assay for the toxic effect of immobilised bifenthrin

The toxic effects of bifenthrin-immobilised and bifenthrin-coated (free group) wood chips were quantified by examining the mortality for each test treatment, as the mortality of the control...
group without bifenthrin was zero. The mortality was averaged according to the number ($n = 3$ in each treatment) and target dose ($1.25, 2.5$ and $7.5 \, \mu g \, cm^{-2}$ bifenthrin). As shown in Fig. 10, the mortality in the bifenthrin-immobilised group shows an apparent statistical difference from that in the bifenthrin-coated free group ($P < 0.001$). For the bifenthrin-coated (free group) wood chips, a dose-dependent increase of mortality becomes evident. For the bifenthrin-immobilised wood chips, however, the termite mortality was low-dose selective, and $1.25 \, \mu g \, cm^{-2}$ bifenthrin appeared to cause the highest mortality, which is favourable for practical application, because this implies that a high dose of the chemical is not necessary and thus contamination of the environment remains minimal.

### 3.4 Bifenthrin release kinetics in vitro

The release kinetics of bifenthrin from several different samples was analysed. For the simple bifenthrin-coated wood chips (free group), the release rate at each time point during a time period of $24 \, h$ is summarised in Fig. 11 and Table 3. It is shown that, over $24 \, h$, the $1.25 \, \mu g \, cm^{-2}$ bifenthrin-coated chip releases $\sim 93.4\%$ bifenthrin. What is very interesting is that no release from the bifenthrin-immobilised wood chip (immobilised group)
was detected, indicating the very preferred specification of the immobilised chips for practical distribution.

4 DISCUSSION
Photochemical modification to immobilise different types of organic molecule on various types of material and the photoimmobilisation method in cell and antibody analysis have been described and reported. By photoimmobilisation, the introduced groups are covalently bound to the container material surface in cell culture, and long-term stability and high activity of the chemicals in the cell culture medium are observed.\(^2^2\)

Recently, surface modification of biomaterials has been extensively performed. For example, a novel histidine-containing polymer with azido groups was photoactively immobilised on a conventional polymer surface to create an antibiofouling material. The results demonstrated that protein adsorption on the polymer-immobilised regions was reduced, and the spreading and adhesion of mammalian cells were also reduced in comparison with those in non-immobilised regions.\(^1^5\) In addition, the photoimmobilisation technique was used for the first time in surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements. Reports showed that various types of biomolecule, including DNA, protein, polysaccharide, lipid, virus and low-molecular-weight chemicals, were immobilised and specifically recognised by the corresponding antibody.\(^2^3\)

Moreover, Gong and Ito\(^1^6\) successfully prepared a multivalent ligand of thrombopoietin (TPO) by immobilising mimetic peptides on a gold particle surface. They found that the multivalent ligand enhanced the growth of TPO-dependent cells by comparison with the monovalent ligand. In addition, the present authors’ preliminary investigation on the anticervical cancer functionality of biomaterials also revealed that coimmobilised IFN-\(\gamma\) plus TNF-\(\alpha\) does induce apoptosis in human cervical cancer cells, and the inhibiting activity to the growth of human cervical cancer cells was higher than the free one.\(^2^0\)

As this technique can be employed for the microarray immobilisation of various biological molecules or components, the present authors synthesised photoactive silk fibroin and used it as the coating layer to fabricate bifenthrin and immobilise it on the surface of wood chips by photolithography, in an attempt to create a polymeric bifenthrin drug that could improve the termitic control strategy in the future.

The present results indicate the successful introduction of 4-azidobenzoic acid into silk fibroin (Figs 5, 6 and 7, Table 1), and the silk fibroin-bifenthrin was immobilised effectively on the wood chip surface (Figs 8 and 9, Table 2). The immobilised bifenthrin maintains high activity against termites (Fig. 10), noticeably higher than that of soluble bifenthrin at a dose of 1.25 \(\mu\)g cm\(^{-2}\) (Fig. 10a). Moreover, the time course of biological activity indicated that the immobilised bifenthrin had a quicker toxic action against termites than soluble ones at a lower dose, for example at a dose of 1.25 \(\mu\)g cm\(^{-2}\) (Fig. 10a). As the immobilised molecules transduce a signal for a longer time than soluble molecules do,\(^2^2\) in the present study the quicker toxic action and the higher activity of the immobilised bifenthrin are probably due to a higher local concentration and long-term signal stability.

Based on the above results, a new application of photoimmobilisation of bifenthrin that may provide a new strategy for termitic control has been demonstrated. The photoimmobilised bifenthrin on cellulose chip was effective and toxic to termites, although the action mechanism of immobilised bifenthrin needs to be further investigated. Unlike free bifenthrin, immobilised bifenthrin does not spread over the environment through rain and subterranean water when applied to treat termites in houses or other constructs. This is the major advantage of the technique.

5 CONCLUSION
In conclusion, a novel photoactive silk fibroin was prepared by the reaction of silk fibroin with N-(4-azidobenzoyloxy) succinimide, which was used to immobilise bifenthrin on the surface of wood (cellulose). The immobilised bifenthrin was active and

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Table 2. Atomic percentage of the surface of modified cellulose\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Atomic percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>64.2 35.8 0</td>
</tr>
<tr>
<td>AzPhSF-cellulose</td>
<td>55.1 31.1 13.6</td>
</tr>
<tr>
<td>AzPhSF-bifenthrin-cellulose</td>
<td>69.6 28.8 1.6</td>
</tr>
</tbody>
</table>

\(^a\) Calculated from ESCA survey scan spectra.

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Figure 10. The termite mortality rate induced by free bifenthrin and photoimmobilised silk fibroin mixed with bifenthrin on cellulose for 8 days: (a) 1.25 \(\mu\)g cm\(^{-2}\); (b) 2.5 \(\mu\)g cm\(^{-2}\); (c) 7.5 \(\mu\)g cm\(^{-2}\). Significant increase at \(*\) \((P < 0.001)\) when compared with the free bifenthrin group. Data represent means \(\pm\) standard deviations \((n = 3)\).
Figure 11. Gas-phase chromatography spectrum of bifenthrin release from a bifenthrin-coated chip in 24 h. The setting time points are 0.5, 1, 2, 3, 4, 5, 6, 7 and 24 h (A to J).

Table 3. Bifenthrin release during 24 h from bifenthrin-coated and bifenthrin-immobilised wood chips

<table>
<thead>
<tr>
<th>Release time (h)</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifenthrin-coated (%)</td>
<td>99.2 (±1.6)</td>
<td>98.2 (±1.8)</td>
<td>96.6 (±1.2)</td>
<td>95.9 (±0.7)</td>
<td>95.2 (±0.0)</td>
<td>94.6 (±1.8)</td>
<td>94.1 (±0.8)</td>
<td>93.7 (±1.2)</td>
<td>93.4 (±2.0)</td>
</tr>
<tr>
<td>Bifenthrin-immobilised (%)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

a Analysed by gas-phase chromatography.

toxic to termites. This technique may provide a new strategy for environmentally benign termite control.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Rong Zheng for improving the manuscript, and Professor Changlian Peng for helpful discussions. Financial support by the Natural Science Foundation of China (30970731, 50832002), the Natural Science Foundation of Guangdong Province (9151063101000015) and the MOE Key Laboratory of Laser Life Science, South China Normal University, is acknowledged.

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