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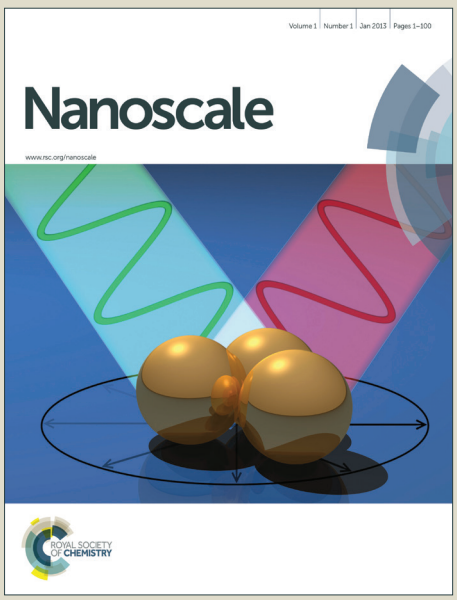

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Functionalized fluorescent dendrimer as pesticide nanocarrier: application in pest control

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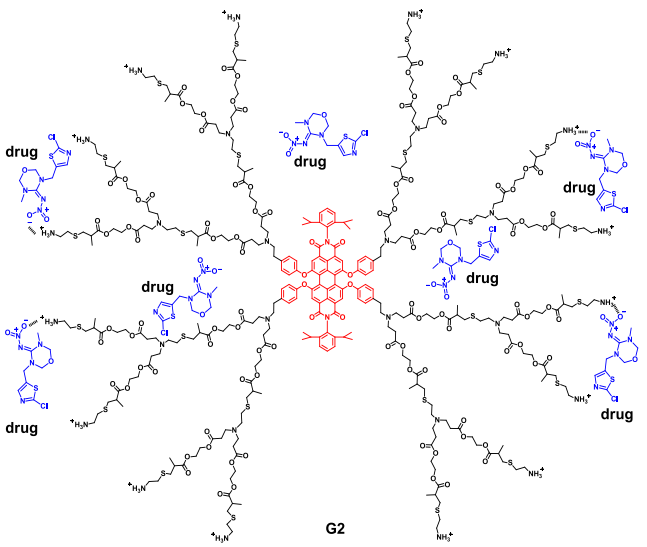
We report the delivery of a hydrophobic pesticide, thiamethoxam, by water-soluble nanosized cationic dendrimers that contain hydrophobic dendritic polyesters and peripheral amines, demonstrated by DLS, spectral analysis and ITC. The dendrimer-based nanocarrier can efficiently deliver pesticide into the live cells and largely increase the cytotoxicity of the drug.

In recent decades, excessive use of chemical pesticide causes pest resistance to insecticide. Meanwhile, the residue of pesticide causes many environmental problems threatening the health of human beings and animals. Thus, there is a great need to explore novel methods for pest control to reduce the side effects of pesticide. Recently some nanocarriers, such as polymeric nanoparticles,[1] liposomes,[2] and dendrimers,[3] have been utilized to deliver and improve the drug effects. Dendrimers are particularly suitable carriers with concomitant possibility for the drug's delivery due to their precisely controllable nanosizes, low polydispersities, and multiply modifiable surface functionalities.[4] Drugs either are covalently conjugated to the peripheral groups of the dendrimer or encapsulated inside dendrimer's cavity. Many drugs have been applied such as methotrexate (MTX),[5] paclitaxel, doxorubicin (DOX), 5-fluorouracil (5-FU), camptothecin and pesticide.[6] Polyester dendrimers have recently been explored as drug carriers with attractive characteristics such as biodegradability, biocompatibility, and immunocompatibility.[7] One of examples is 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) polyester dendrimers based nanoparticles which shows an excellent capacity for the encapsulation and controlled release of a hydrophobic anticancer drug DOX.[7a] However, a majority of pesticides are hydrophobic and lack solubility in physiological solutions, which can greatly impair their cellular internalization and lead to low efficacy.[8] As a result, organic solvents such as dimethyl sulfoxide (DMSO) are often used to dissolve pesticides. While these organic solvents are high cytotoxic and thus nonuse of organic solvents would be desirable.

The functionalized cationic dendrimer-based nanocarriers (G1, G2, and G3, Scheme S1) consist of a fluorescent perylene-3,4,9,10-tetracarboxydiimide chromophore (PDI) in the center and precise amino groups at the periphery (Scheme 1). The central PDI chromophore is a popular dye and pigment due to its excellent chemical, thermal, and photochemical stability, which allows the detection via fluorescence microscopy.[9] The outer dendritic polyester units are biodegradable and biocompatible with very low toxicity and low immunogenicity[7b] and also provide the cavities for the delivery of hydrophobic drug. The peripheral amino groups contribute to the water solubility of the dendrimers. Previous study demonstrated that these fluorescent cationic dendrimers can be rapidly engulfed into live insect cells with high gene transfection efficiency as well as low cytotoxicity.[10] Functionalized PDI derivatives have been developed as efficient carriers for DNA and dsRNA[11] but not yet for drugs so far. The previously reported dendrimers were water-soluble, biocompatible, and non-fluorescent character.[3d, e, 5, 7a, 12] Therefore, it would be of high interest to explore the fluorescent PDI-cored dendrimers as drug nanocarriers.



Scheme 1. Schematic interaction between nanocarrier G2 and the drug thiamethoxam.

In this paper, we report the delivery of a hydrophobic drug, thiamethoxam, by water-soluble PDI-cored cationic dendrimers (G1, G2, and G3). The thiamethoxam is an insecticide in the class of neonicotinoids and has pesticidal activity against homoptera pests

such as aphids, leafhoppers and planthoppers.[13] The chemical structure of the drug is shown in Scheme 1. The drug shows relatively low water solubility (4.1 mg/mL), but high solubility in organic solvents.[14] By mixing the complex of nanocarrier/drug into the insect's diet, the mortality of the pests was largely elevated in comparison with the control experiments. Therefore, a novel method has been successfully explored so that the water-soluble PDI-cored cationic dendrimers act as efficient drug nanocarriers to increase the drug cytotoxicity for pest control.

To explore an efficient carrier for drug delivery *in vivo*, we preliminary screened various kinds of gene carriers and chose the cationic dendrimers (G1, G2, and G3, Scheme 1) for further investigation. The water-soluble PDI-cored cationic dendrimers were synthesized from 2-methacryloyloxyethyl acrylate and cysteamine using the sequential sticking method. Detailed synthesis procedures and material characterizations can be found in the literature.[10] The maximum absorbance and emission of the dendrimers are about 590 and 615 nm, respectively. The synthesis of first generation dendrimer (G1) needs two steps. The higher generations (G2 and G3) have more cavities and peripheral cations but need more synthesis steps and longer reaction periods than G1. In our previous work, all the three dendrimers (G1, G2, and G3, Scheme S1) can enter into live cells. G2 and G3 are detectable inside the cells after 1 h incubation, while G1 requires 2 h incubation (Fig. S1), demonstrating that the cellular internalization of G2 and G3 is faster than that of G1.[10]

The sizes of these dendrimer-based nanocarriers and nanocarrier/drug complexes were investigated by dynamic light scattering (DLS). The sizes of G1, G2, and G3 dendrimers were 1.1 nm, 1.8 nm, and 3.2 nm, respectively (Table 1).[10] After the interaction of hydrophobic insecticide, thiamethoxam, the mean sizes of drug/dendrimer complexes were increased to the range of 166-178 nm (Table 2). It implies that the interaction can be attributed to the hydrogen bonds and hydrophobic interactions.[15] The appropriate sizes of the complexes are below 200 nm, which implies that the sizes of the nanoparticles are suitable for *in vitro* and *in vivo* applications.[12, 16]

Table 1. Dynamic light scattering (DLS) data of G1, G2, and G3 in water.

molecule no.	number of $NH_3^+Cl^-$ groups	radius (nm) in water
G1	8	1.1 ±0.5
G2	16	1.8 ±0.3
G3	32	3.2 ±0.2

Table 2. The complex sizes of dendrimers and drug (Dendriplex) in water.

Dendriplex no.	Average size (nm)	Polydispersities
G1/drug	175.4 ±1.9	0.38
G2/drug	166.0 ±1.6	0.23
G3/drug	178.6 ±2.1	0.27

We then assayed the interaction between the nanocarriers and the insecticide by spectral analysis. Figure S2 shows that the addition of the drug into G2 solution does not lead to obvious change in the absorption spectra. Interestingly, upon the addition of drug, the fluorescence intensities of G2 gradually increase accompanied by a 3 nm blue shift of the peak wavelength (Fig. 1(A)). The enhancement of fluorescence intensity suggests the non-covalent molecular interactions, which might be hydrogen bonds and hydrophobic interactions between the dendrimer G2 and drug (Scheme 1). When the drug concentration reach 400 μM, the hyperchromicity of G2 and G3 are about 225%, and the hyperchromicity of G1 is 192% (Fig. S3), demonstrating that G2 and G3 have much potential to interact with the drug than G1.

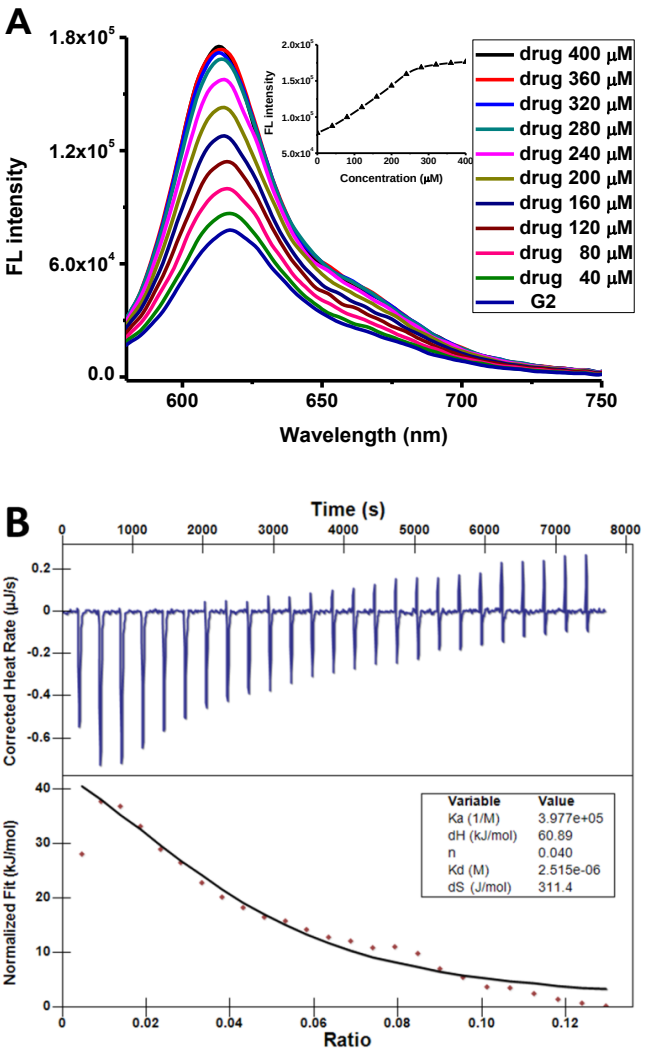


Fig. 1 (A) Changes in fluorescence intensity of G2 with the addition of drug (thiamethoxam, 0 μM to 400 μM) in water at 25 °C (Ex = 545 nm). (B) ITC titration of G2 (syringe, 65 μM) into thiamethoxam solution (cell, 138 μM). Binding isotherm (heat change versus G2/thiamethoxam molar ratio) is obtained from the integration of raw data (bottom).

To further investigate interactions of nanocarriers (G1, G2 and G3) with thiamethoxam, the thermodynamic parameters of dendrimer/thiamethoxam were quantified by isothermal titration calorimetry (ITC). The acquired titration curves and thermodynamic parameters are given in Fig. 1(B), Fig. S4, and Fig. S5. The binding constant (K_a) of G2/thiamethoxam (3.98×10^5 M^{-1}) and G3/thiamethoxam (5.45×10^5 M^{-1}) are higher than that of G1/thiamethoxam (2.07×10^5 M^{-1}), suggesting that G2 and G3 more effectively interact with drug than G1. This is consistent with the results obtained from the above spectral analyses. It shows that all $\Delta H > 0$ and $\Delta G < 0$ (Fig. 1(B), S4 and S5). The negative value of ΔG reveals an automatic reaction occurs between nanocarrier and thiamethoxam. The positive enthalpy (ΔH) shows that the non-covalent molecular interactions, such as hydrogen bonds and

hydrophobic interactions, play important roles in the interaction of thiamethoxam with nanocarriers.[17] Therefore the interactions between nanocarriers and thiamethoxam have been convincingly demonstrated and this is consistent with our prediction.

To explore whether these nanocarriers can deliver drug and apparently increase the drug cytotoxicity to insect pests, live insect cells were first treated with the nanocarrier/drug complexes. It has been reported that these nanocarriers-treated cells exhibit >92% viabilities at various concentration.[10] We then evaluated the cytotoxicity of 0.5 μM nanocarrier complexed with various amount of thiamethoxam, using the Tali™ cell viability assay. After 48 h incubation, the statistic effect of the nanocarrier/drug complexes on culture cells showed apparent enhanced cytotoxicity (Fig. 2). Reasonably, the cell mortalities were increased along with the addition of more insecticide. Higher generation of nanocarriers showed larger enhancement of cytotoxicity than lower generation of nanocarriers. While, without the support of nanocarriers, drug alone exhibited much lower cytotoxicity. These results indicate that all the nanocarriers (G1, G2, and G3) can efficiently deliver insecticide into live insect cells. The elevated cellular internalization of the drug thus enhances its cytotoxicity.[3d]

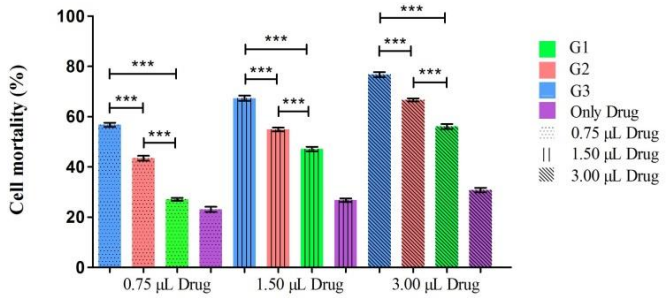


Fig. 2 Cytotoxic assays. Cell mortality of live insect cells treated with nanocarrier/drug complexes. Nanocarrier G1, G2 and G3 at the concentration of 0.5 μM deliver the various dosage of drug: 0.75 μL, 1.5 μL and 3 μL, respectively. The cells treated with drug alone serve as a control. Means ± SEM with *** are significantly different (pairwise comparison of t-tests, $p<0.0001$).

The rapid cellular internalization of drug carriers is prerequisite for the *in vivo* application. For the subsequent *in vivo* test on live insects, the relatively simple synthesis procedure and the above satisfied cytotoxicity enhancement give the intermediate nanocarrier, G2, a good choice for the future potential application for insect control. Therefore, we first performed an *ex vivo* test by choosing G2 as a representative case in live tissues. Herein, various tissues of larval insect, *Heliothis armigera* which is one of the most destructive pests on many crops,[18] were dissected and cultured for 1 h in a standard culture medium containing 10 μM G2, and then washed with the PBS buffer for 1 h. G2 fluorescence was observed in all tested tissues by fluorescence microscopy, as shown in Fig. 3. These results demonstrate that G2 can efficiently enter into all tested insect tissues. Then we further tested whether oral feeding of G2 can efficiently enter into larval gut cells. To this end, first instar larvae were fed with fresh artificial diet containing 24 μg G2, while normal artificial diet was used as a negative control. After 3 days of oral feeding, the larvae were dissected to obtain guts. The fluorescence of G2 can be detected in gut cells by fluorescence microscopy (Fig. 4), demonstrating that G2 successfully passes through the peritrophic membrane and efficiently enters into the gut cells. In addition, the diet containing G2 failed to show any effect on the development of the larvae. All tested larvae survived to adulthood and generated normal offspring. It would be desirable to directly visualize the distribution of nanocarrier/drug complexes in the larval tissues. Unfortunately, there is no traceable tool to label the drug. Therefore, the application of G2/drug complexes showed same fluorescent distribution as G2 alone and was not shown. These results demonstrate that the G2 fulfills the application of drug carrier *in vivo* application on the insect model.

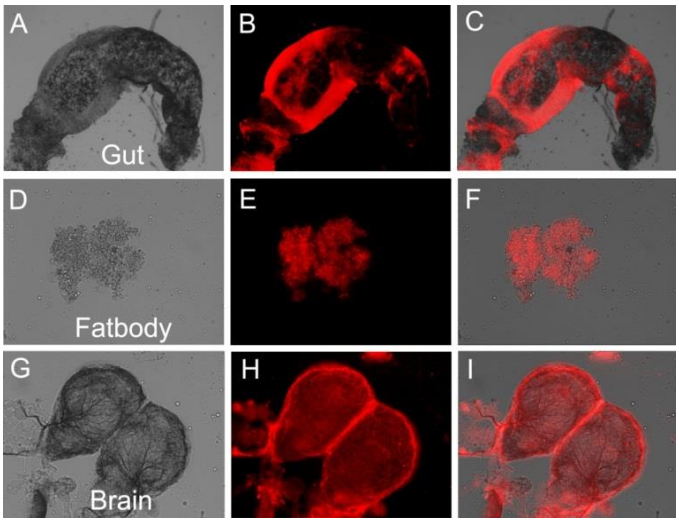


Fig. 3 G2 efficiently enter into various larval tissues. Fluorescence images of G2 (B, E, H) internalized into dissected live tissues of gut (A), fatbody (D), and brain (G), merged channels are shown (C, F, I).

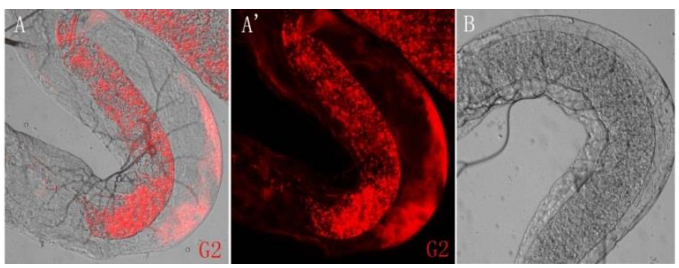


Fig. 4 Fluorescence images of dissected live gut. (A) Insects are fed with artificial diet containing G2. (A') Separated channel to show G2 engulfed by gut cells. (B) Normal diet as a negative control.

In the next step, we simply mixed G2 with drug in solution, and then added the mixture to the diet of 2nd instar larvae, while H_2O/drug was used as the control. The drug, thiamethoxam, is able to kill some homoptera pests but not *Heliothis armigera*.[13] As expected, in the control experiment, the mortality of *Heliothis armigera* larvae fed with H_2O/drug complexes is less than 20% after 5 days of treatment (Fig. 5 (A)). Excitingly, the mortality of larvae fed with G2/drug complexes reached 50% on the second day's feeding and was further increased to 66.7% and 83.3% on the third and the fourth day's feeding, respectively (Fig. 5 (A)). It was noticeable that more than 90% larvae fed with G2/drug complexes died on the fifth day's feeding (Fig. 5 (B)). The larvae fed with G2/drug complexes clearly showed the phenotypes with severe defects in development and their body sizes were dramatically reduced compared with the controls (Fig. 5 (C)), indicating that the drug toxicity is largely elevated after complexing with G2. Therefore, the drug thiamethoxam, which is not able to kill *Heliothis armigera* under normal application probably due to the low efficiency of cellular internalization of the drug, now shows strong pesticidal effect against this kind of pest under the help of G2-mediated cellular internalization. These results are consistent with the

cytotoxicity assays on live cells in which the cell viability is largely decreased in the treatment of drug complexed with G2 nanocarrier (Fig. 2). Finally, G2 is successfully explored as an effective pesticide nanocarrier to dramatically enhance the effect of the pesticide and to kill the nontarget pests.

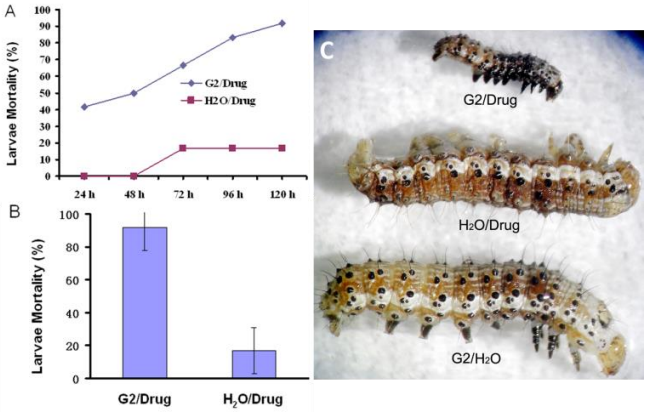


Fig. 5 (A) G2/drug-fed larvae die at higher penetration than the control at all treated durations. (B) More than 90% G2/drug-fed larvae are died after 5 days treatment. Error bars indicate the standard deviation. (C) G2/drug complexes suppress larval development. Larvae were fed with G2/drug, H_2O/drug, and G2/H_2O contained diet, respectively.

Conclusions

In summary, a novel application of water-soluble fluorescent PDI-cored cationic dendrimers is reported as efficient drug nanocarriers. The fluorescent cationic dendrimer-based nanocarriers consist of a central PDI chromophore and dendritic hydrophobic polyesters and peripheral amino groups. A hydrophobic drug, thiamethoxam, can be effectively delivered by the fluorescent dendrimer-based nanocarriers and their molecular interaction was assayed using DLS, spectral analysis and ITC. The dendrimer-based nanocarriers can efficiently deliver the drug into the live cells and largely increase the cytotoxicity of the drug. Using a nontarget pest of this drug, by incorporating the mixture of nanocarrier/drug complexes into the diet of the pests, the mortality of the fed larvae was efficiently elevated compared with that of the control experiments. Therefore, the dendrimers are successfully explored as pesticide nanocarriers to enhance the cytotoxic effect of the pesticide. This is the first example of PDI-core fluorescent dendrimers applied as pesticide nanocarriers and also is the first application on insect model. This novel application can efficiently increase the pesticide effects and extend insecticidal target pests, which will open a new avenue to avoid the overuse of chemical insecticides.

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