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Short communication

Evaluation of penetration of nanocarriers into red pepper leaf using confocal laser scanning microscopy

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ABSTRACT

Penetration of nanocarriers into red pepper leaf was evaluated with the aim of identifying a novel way to enhance the efficacy of applied pesticides on the field. In addition, the effect of surface charge of the nanocarriers on penetration was also studied. Particularly, corn oil-nanoemulsions (NE) and chitosan coated NE (CH-NE) were successfully prepared with a high encapsulation efficiency, a high payload, a very small size, a small polydispersity index, and a high zeta potential. However, after being coated with chitosan, the zeta potential of NE changed from a negative charge to a positive charge. Penetration experiments were also carried out using a Franz diffusion cell followed by visualization using confocal laser scanning microscopy. The images of the vertical sections illustrated that penetration of nanocarriers (NE and CH-NE) into the red pepper leaf occurred very quickly. Nanocarriers fully penetrated the whole leaf after 60 min. Moreover, multi-depth images that paralleled the leaf surface (horizontal visualization) together with removal of plant-autofluorescent emissions and three-dimensional graphs describing the penetration of NE and CH-NE demonstrated that the negatively charged nanocarriers (NE) had a higher penetration rate compared to that of the positively charged nanocarriers (CH-NE).

1. Introduction

Pesticides play an important role in agriculture, as they help prevent crop loss caused by insect pests (Pimentel, 1995; Gilden et al., 2010). However, in the natural environment, pesticides can be dispersed into the air and water and can be degraded through photodegradation, biodegradation, and hydrolysis (Tiryyaki and Temur, 2010; Liu et al., 2010). Therefore, only a small amount of pesticides (<0.1%) actually reaches target pests (Pimentel, 1995; Wang and Liu, 2007). The remainder can adversely affect non-target organisms such as through fish kills, disturbed avian reproduction, and human illness (Kromer et al., 2004; Covaci, 2006; Arias-Estévez et al., 2008). Foliar uptake is an effective way to apply pesticides to enhance their efficacy and minimize environmental pollution (Wang and Liu, 2007). However, pesticides must be available on both sides (top and bottom) of the leaf as well as maintain their killing effect for a sufficient time for better protection of plants from chewing and sucking insects (Wang and Liu, 2007).

Nanocarriers in their various forms have been investigated for diverse industrial applications, including the cosmetic, agriculture, pharmaceutical and food industries due to their small size (10–1000 nm), higher surface area to volume ratio, high payload of pesticide, high photo-protection capacity, and controlled release of encapsulated pesticides (Bang et al., 2009; Mishra et al., 2010; Nguyen et al., 2012, 2013). As a result, nanocarriers have been used to form safer pesticide formulations, avoid repetitive applications or the need for higher doses, and reduce the risk of contaminating water and soil while maintaining the desired effect on the target (Frederiksen et al., 2003; Nguyen et al., 2013). Moreover, nanocarriers possess a good ability to penetrate through animals and human skin (Sinico and Fadda, 2009; Aripin et al., 2013). On the other hand, there are a lot of stomata on both sides

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of leaves, and size of stomatal apertures is from a few microns up to several microns (Wang et al., 2014). Consequently, passing through stomatal aperture is also an easy pathway for nanocarriers to deeply penetrate into leaf because their particle size is many times smaller than stomatal apertures. However, a practical study about penetration of nanocarriers through leaf and effect of their surface charge (zeta potential) on the penetration as well as visualizing their penetration using confocal laser scanning microscopy has not been reported. Therefore, in this study, the penetration into leaf of nanocarriers encapsulating pesticides and the effect of their surface charge on penetration will be investigated using confocal laser scanning microscopy. Particularly, deltamethrin, a synthetic type II pyrethroid insecticide and one of the most potent insecticides known (Ding et al., 2004), and Nile Red were encapsulated into corn oil-nanoemulsions as an active ingredient and a fluorescent dye, respectively. Penetration of the prepared nanoemulsions into red pepper leaves was carried out using a Franz diffusion cell followed by vertical and horizontal visualization using confocal laser scanning microscopy. Furthermore, the effect of surface charge (negative or positive) of the nanocarriers on penetration through red pepper leaves was also compared.

2. Materials and methods

2.1. Materials

Red pepper seedlings (*Capsicum annuum L.*) were purchased from a local market and planted at the Laboratory of Biopolymers Engineering, Korea University (Seoul, Korea).

Lecithin from soybean (Junsei Chemical, Tokyo, Japan) and Tween-80 (Samchun Pure Chemical, Seoul, Korea) were used as surfactants. Corn oil (Sigma–Aldrich, St. Louis, MO, USA) was used as the liquid lipid. Deltamethrin (98%), which is a pyrethroid ester insecticide, was obtained from Kyung-Nong (Kyungju, Kyungsangbuk-do, Korea). Chitosan (molecular weight 30,000 Da, degree of deacetylation: 90–94%) was obtained from Biotech (Mokpo, Jellanam-do, Korea). Nile Red (Sigma–Aldrich) was used as the fluorescent dye. All other chemicals were of analytical grade.

2.2. Preparation of corn oil-nanoemulsions (NE) and chitosan-coated NE (CH-NE)

NE was prepared using a hot high pressure homogenization technique (Lacerda et al., 2011). Briefly, corn oil (5%, w/w) and deltamethrin (2.3%, w/w) were heated to 85 °C, followed by mild stirring to dissolve the deltamethrin. At the same time, a mixture of soybean lecithin and Tween-80 (2.5% w/w with a 1:1 ratio, w/w) was added to distilled water and stirred at 85 °C for 45 min. The surfactant mixture was poured into the corn oil containing deltamethrin. The lipid phase was emulsified in the aqueous phase using an Ultra-Turrax® T 25 basic homogenizer (IKA®-Werke, Staufen, Germany) at 19,000 rpm for 5 min. The pre-emulsion was homogenized during three cycles at 1500 bar using a high pressure homogenizer (model M-110P; Microfluidics, Newtown, MA, USA). The resulting dispersion was mildly stirred for 10 min at room temperature to stabilize the nano-emulsion formulation.

For visualization under confocal laser scanning microscopy, a mixture of Nile Red and deltamethrin at a 1:200 (w/w) ratio was encapsulated into the nanoemulsions.

To prepare CH-NE, chitosan were dissolved in 0.5% (v/v) acetic acid aqueous solution by stirring at room temperature to form a 1% chitosan solution (w/v). Then, the chitosan solution was added dropwise to primary NE dispersions at a 1:10 ratio (v/v) with stirring at room temperature for 20 min.

2.3. Measurement of particle size and zeta potential

Mean size, polydispersity index (PDI) and zeta potential (charge on the surface) of the nanocarriers (NE and CH-NE) were determined using a nano size analyzer (Malvern Zeta sizer, Nano Z-S; Malvern Instruments, Malvern, UK). Measurements were carried out at 25 °C with a detector angle of 90°.

2.4. Encapsulation efficiency and payload

The nanocarrier dispersions were filtered using a cellulose ester membrane with a 1 μm pore size to remove the unencapsulated deltamethrin that had precipitated in solution (Nguyen et al., 2012). The dispersions were diluted 10 times with distilled water followed by dissolving in tetrahydrofuran at a 1:3 ratio (v/v). The encapsulated deltamethrin was detected using reverse-phase high performance liquid chromatography with a model 2690 pump (Waters, Milford, MA, USA), a model 996 photodiode array detector (Waters), and a Kromasil C18 column (250 mm × 4.6 mm) (EKA Chemicals AB, Bohus, Sweden), packed with 5 μm diameter particles. The mobile phase was a mixture of acetonitrile and water (90:10, v/v). The flow rate was 1 mL/min at room temperature. The detection wavelength of deltamethrin was 230 nm. Encapsulation efficiency and payload were calculated based on the following equations:

$$\text{Encapsulation efficiency } (\%) = \frac{\text{Deltamethrin in nanoparticles (mg)}}{\text{Initial added deltamethrin (mg)}} \times 100 \quad (1)$$

$$\text{Payload } (\%) = \frac{\text{Deltamethrin in nanoparticles (mg)}}{\text{Amount of nanoparticles (mg)}} \times 100 \quad (2)$$

2.5. Visualization of nanocarrier penetration into red pepper leaf using vertical sections

Penetration of the nanocarriers through red pepper leaf was performed using a vertical modified amber glass Franz diffusion cell (Daihan Labtech, Gyeonggi-do, South Korea) with an effective diffusion area of 2.27 cm^2 and receptor volume of 15.9 mL. Briefly, after harvesting from the tree, mature red pepper leaves were washed three times with distilled water and cut into a circular shape with a diameter of 2.7 cm. The prepared leaf sample was placed between the donor and receiver compartment of the Franz diffusion cell and then securely fastened with a clip. The receiver compartment was filled with distilled water and stirred continuously at 600 rpm and 30 ± 1 °C. The leaf sample was equilibrated for 20 min. Then, 3 mL of the prepared nanocarriers (NE or CH-NE) containing Nile Red and deltamethrin was applied to leaf surface. After a specified amount of time (30, 60, 90, or 120 min), the leaf sample was removed from the Franz diffusion cell and washed