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Proceeding Paper

Physicochemical Characterization and Effect of Additives of Membrane Vesicles from *Brassica oleracea* L. to Be Used in Nanofertilization [†]

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Abstract: Traditional fertilizers and their intensive use cause different environmental problems and new strategies are necessary to deal with these aspects. In these sense, foliar nanofertilization is a new technology postulate as one of the most promising to use in the near future. This type of fertilization has many environmental advantages but there are different factors that its necessary to solve, as they need to be compatible with other additives. Membrane vesicles derived from plant material have been showed in preliminary studies by their great potential as nanocarriers of different micronutrients such as iron (Fe) or boron (B). A complete optimization of fertilizer system based on nanocarriers to encapsulate different elements from different approach is key to obtaining a suitable and profitable from an economic point of view. In this work, different physicochemical parameters such as size, potential Z or osmotic water permeability were measured in membrane vesicles obtained from Brassica oleracea L. to check the integrity of vesicles for further biotechnological application. Besides, different additives (polyether-modified-polysiloxane [PMP], Tween-20 and polyethylene glycol [PEG]) were added to vesicles at concentration of application to determine an effect in the integrity and functionality of the membranes. The results show that functionality of membrane vesicles was only reduced with polyether-modified-polysiloxane [PMP], but not altered by the rest of the additives. These analyses serve to support subsequent research to advance the implementation of this nanotechnology.

Keywords: agriculture; nanocarriers; nanofertilization; surfactants

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1. Introduction

Recently, there is a global pressure to achieve efficient agricultural technologies, in which an improvement of nutrients uptake is the main goal to minimize abiotic stress and enhance yield [1,2]. In this sense, on the one hand foliar fertilization avoid soil penetration and biodegradation problems [3]. On the other hand, the use of nanocarriers such as liposomes provide an enhancement of efficiency of foliar applications [4], which depends on fertilizer absorption and mobility. Both later requirement often fail in traditional foliar fertilization, since they are highly dependent on relative humidity and temperature [5]. In this field, different vesicles are key to take into account to get suitable system to use in an efficient foliar fertilization, such as the external charge or size. A small size for vesicles encapsulating different micronutrients is necessary since the area of contact with the leaf surface is higher enabling the entry through the stomatal pore [6,7]. The use of nanocarriers from natural sources are being investigated during the last years. This type of nanosystems are biodegradable and different works have been show promising results to use them in novel agricultural technologies [4,6]. In this way, membrane vesicles (proteoliposomes) from plant material, specifically from *brassicas* have been tested as carrier of micronutrients. Zinc (Zn) was encapsulated with high efficiency in this type of membrane vesicles and the delivery of Zn into protoplast was reported [8]. Besides, an increase in the penetrability through stomatal pores for B and Fe was showed when micronutrients were encapsulated in vesicles [6]. These facts can be enhanced with the use of surfactant to modify the vesicle surface and therefore there is an effort to study and screening different surfactants to added to membrane vesicles to use in the delivery of mineral nutrients. In medical areas, many advances have been reached in the modification of liposomes surface for example with polyethylene-glycol (PEG), which conjugated to the surface, increase the targeting to specific tissues [9]. Nevertheless, in agriculture there are limited results in this aspect.

In this study we sought to characterize physico-chemically membrane vesicles, measure the stability of the vesicles over time and determine the effect of different surfactants (Tween-20, PEG and PMP) in osmotic water permeability (*Pf*) as parameter that reflect the functionality of the membrane vesicles. This research supposes a preliminary screening, which gives rise to deepen in this sense to characterize and find the optimal nano-system to use in foliar fertilization.

2. Materials and Methods

2.1. Materials

Inflorescences *Brassica oleracea* L. var. botrytis inflorescences were collected from a commercial farm sited in the Region of Murcia (Lorca, Murcia, Spain).

2.2. Membrane Vesicles Isolation

Cauliflower inflorescences were cut into small pieces before vacuum-filtering, at a 1:1.6 (*w*/*v*) ratio, with an extraction buffer (0.5 M sucrose, 1 mM DTT, 50 mM HEPES, and 1.37 mM ascorbic acid, at pH 7.5) and 0.6 g of Polyvinylpyrrolidone (PVP). The mixture was homogenized using a blender and filtered through a nylon mesh (pore diameter of 100 µm). The filtrate was centrifuged (10,000× *g*, 30 min, 4 °C) and the supernatant was recovered and ultracentrifuged (100,000× *g*, 35 min, 4 °C). The pellet obtained was suspended in FAB buffer (5 mM PBS and 0.25 M sucrose, pH 6.5) for storage at −80 °C. The protein concentration was determined by the Bradford method [10].

2.3. Particle Size, Zeta Potential, and Polydispersity Index Analysis of Membrane Vesicles

Dynamic light scattering (DLS) was used to detect particle size, zeta potential, and polydispersity index at a temperature of 20 °C using a Zetasizer Nano (Malvern Instruments, Malvern, UK) in a similar way as previously was reported [11].

2.4. Samples Preparation

Membrane vesicles were used at 0.05% (*w*/*v*) of protein and different surfactant were mixed with vesicles at different concentrations. Tween-20 and polyethylene glycol (PEG) were added at 1% and 2% (*w*/*v*) and polyether-modified-polysiloxane (PMP) was tested at 0.1% (*w*/*v*).

2.5. Stopped-Flow Light Scattering

The osmotic water permeability (Pf) was measured as the velocity of the volume adjustment of the membrane vesicles after changing the osmotic potential of the surrounding medium. The volume of the vesicles was followed by 90° light scattering at λex = 515 nm. The measurements were carried out at 20 °C in a PiStar-180 Spectrometer (Applied Photophysics, Leatherhead, UK), as described previously [12]. Pf was computed from the light scattering time course, according to the following equation:

$$Pf = k_{exp}\ V_0/A_v\ V_w\ C_{out}, \quad (1)$$

where kexp is the fitted exponential rate constant, V0 is the initial mean vesicle volume, Av is the mean vesicle surface area, Vw is the molar volume of water and Cout is the external osmolarity.

2.6. Statistical Analysis

The statistical analyses were carried out using IBM SPSS Statistic 27 for Windows. Anova one-way followed by Tukey's HSD test at the $p < 0.05$ was chosen to determine significant differences between treatments. Small letters on top of bars point the significant differences between treatments.

3. Results

3.1. Physico-Chemical Characterization

Physico-chemical measurements were carried out with different population of vesicles. A selection by size was made by filtration with the objective of provide suitable vesicles with different sizes depend of final application. Three vesicle populations were determined, one with average hydrodynamic diameter of 400 nm without filtration, and other two with lower average hydrodynamic diameters after filtration by 0.45 µm (355 nm) and by 0.22 µm (200 nm) (Figure 1a, bar charts). Polydispersity indexes about 0.4 did not change with the different filtrations (Figure 1a, line). In the same way, zeta potential values neither were modified with the filtrations and a negative value about −30 mV was stablished for membrane vesicles (Figure 1b).

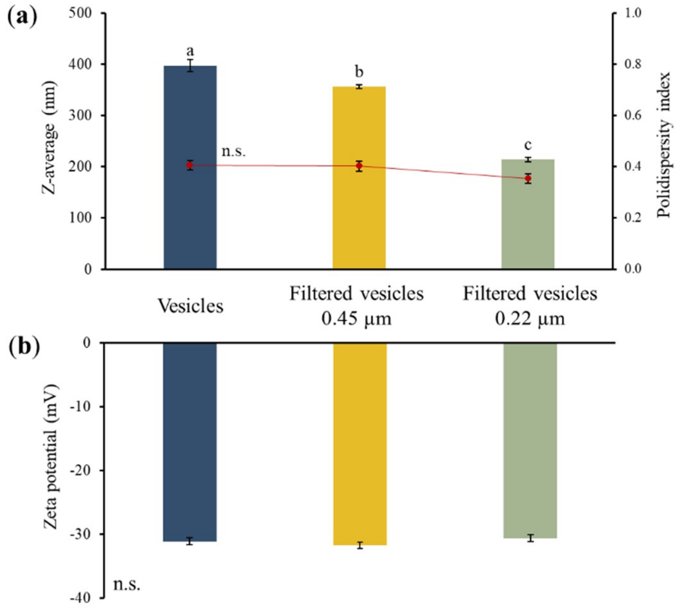

Figure 1. Physicochemical parameters membrane vesicles. (**a**) Z-average (nm) and polydispersity index and (**b**) Zeta potential (mV) of vesicles, vesicles filtered by 0.45 µm and 0.22 µm. Data are means ± SE (n = 3).

3.2. Stability of Vesicles Functionality over Time

Functionality and stability of membrane vesicles were determined through osmotic water permeability value (*Pf*) over time. An initial value about 25 µm s^{-1} was kept for 18 months when vesicles were stabilized with polyalcohol (Figure 2).

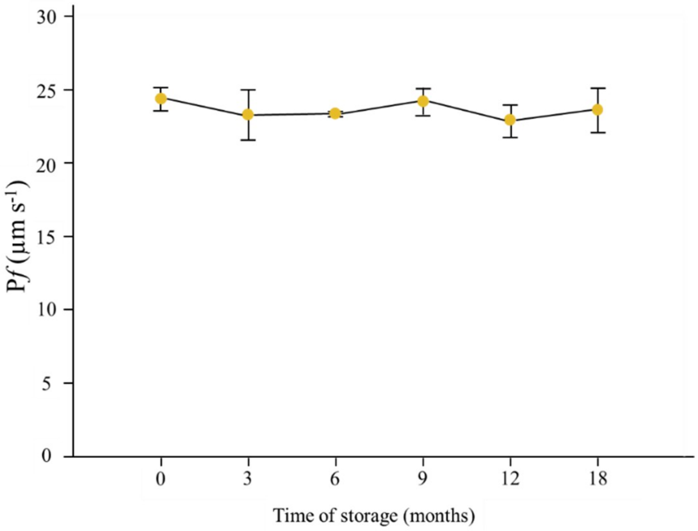

Figure 2. Membrane vesicles stability. Time course (months) of the osmotic water permeability (Pf) of membrane vesicles at 20 °C resuspended in potassium phosphate buffer and stabilized with polyalcohol. Data are means ± SE (n = 30).

3.3. Effect of Surfactant in Membrane Vesicles Functionallity

Once stability over time of membrane vesicles functionality was checked, different surfactants were added to the vesicles to determine if the osmotic water permeability was altered as a control parameter of the functionality. Table 1 display *Pf* values measured in membrane vesicles alone (control) and with Tween-20 and PEG at two concentrations (1 and 2%) and with 0.1% PMP. Results showed only PMP modified *Pf* of membrane vesicles, which was reduced by about 30%.

Table 1. Osmotic water permeability values of membrane vesicles with surfactants at different concentrations. Data are means ± SE (n = 30).

Applied Surfactant in Membrane Vesicles	***Pf* (μm s^{-1})**
Control	22.5 ± 2.8
1% Tween-20	21.8 ± 1.3
2% Tween-20	23.6 ± 2.4
1% PEG	25.4 ± 5.0
2% PEG	22.8 ± 3.1
0.1% PMP	15.0 ± 1.2 *

PEG, Polyethylene Glycol; *Pf*, osmotic water permeability; PMP, polyether-modified-polysiloxane.

4. Discussion

Foliar fertilization is a potential area to develop new technologies, for example focused on nanocarriers [6,13]. However, it is still necessary to deepen the characterization and improvement of the nano-systems to reach the higher efficiency. Proteoliposomes from natural sources such as *Brassica* plants have been show for their potential use in different biotechnological applications, encapsulating bioactive compounds and delivery them in animal cells [14,15]. Besides, some studies have been carried out with an agricultural approach [6,8]. Based on these previous research, membrane vesicles from *Brassica oleracea* L. var. botrytis inflorescences were used to advance research to find the optimal nano-systems.

Different population of membrane vesicles with different average size were obtained by filtration. Size is an important characteristic in nanobiotechnology and get the optimal size could be key in the further applications. Size between 400 and 200 nm were obtained without modify the homogeneity (PdI) and the charge (Zeta potential) of the samples.

These sizes are suitable to be use in different applications [16], including foliar application, in which an entry through the stomatal pore is possible, since this has a range of 500–100 nm [7].

Vesicles based on natural membranes are made up of both lipids and proteins. A characteristic of these vesicles is their capacity to pass water through membrane and therefore the osmotic water permeability value (*Pf*) could be a parameter to determine the functionality and integrity of the membrane vesicles. This value was measured in storage membrane vesicles stabilized with polyalcohol for 18 months and vesicles functionality was confirmed after storage time. Possibility to preserve the integrity of the nano-systems is essential to use them in a final application, for example, when incorporating them as commercial fertilizer, since a long shelf-life is required.

Modification of vesicle surface is key to improve the characteristic of de nano-systems. Addition of surfactants could be interesting to increase the shelf-life or the capacity of vesicles to have a specific target where delivery the cargo (micronutrients in this case) [17]. In this sense, different surfactants were tested in order to know if *Pf* of membrane vesicles was altered when surfactant were added and only with PMP the *Pf* value decreased. To determine the cause of *Pf* decrease with PMP would need more investigation in relation with aquaporins, since these proteins are transmembrane channel involved in the water passage through membranes. Besides, these proteins were related to the stability of membrane vesicles from broccoli in a previous study [18].

5. Conclusions

In this study, we have shown that membrane vesicles from *Brassica* plants have suitable physico-chemical characteristics and stability over time to be used in biotechnological applications, such as new technologies in agriculture. Besides, results obtained from surfactant screening opens a new area of research, the modification of the surface of membrane vesicles by surfactants with the aim of improving shelf life and cargo targeting, and efficiency since some of surfactant tested did not altered the functionality of vesicles.

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