biology and life sciences forum

MDPI

Proceeding Paper

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

Lucía Yepes-Molina *, Juan José Ríos and Micaela Carvajal

Group of Aquaporins, Plant Nutrition Department, Centro de Edafologia y Biologia Aplicada del Segura, CEBAS-CSIC, Campus Universitario de Espinardo, E-30100 Murcia, Spain; jjrios@ugr.es (J.J.R.); mcarvaja@cebas.csic.es (M.C.)

* Correspondence: lyepes@cebas.csic.es

† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: Traditional fertilizers and their intensive use cause different environmental problems and new strategies are necessary to deal with these aspects. In this sense, foliar nanofertilization is a new technology postulated as one of the most promising for use in the near future. This type of fertilization has many environmental advantages but there are different factors that require a solution, as it needs to be compatible with other additives. Membrane vesicles derived from plant material have been shown in preliminary studies to have great potential as nanocarriers of different micronutrients such as iron (Fe) or boron (B). A complete optimization of the fertilizer system based on nanocarriers encapsulating different elements from different approaches is key to obtaining a system that is 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. Furthermore, different additives (polyether-modified-polysiloxane [PMP], Tween-20 and polyethylene glycol [PEG]) were added to vesicles at different concentrations of application to determine the effect on the integrity and functionality of the membranes. The results show that the functionality of the 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

Citation: Yepes-Molina, L.; Ríos, J.J.; Carvajal, M. Physicochemical Characterization and Effect of Additives of Membrane Vesicles from *Brassica oleracea* L. to Be Used in Nanofertilization. *Biol. Life Sci. Forum* **2022**, *11*, 21. https://doi.org/10.3390/IECPS2021-11954

Academic Editor: Dimitris L. Bouranis

Published: 30 November 2021

1. Introduction

Recently, there has been global pressure to achieve efficient agricultural technologies in which an improvement of nutrient uptake is the main goal to minimize abiotic stress and enhance yield [1,2]. In this sense, on the one hand, foliar fertilization avoids soil penetration and biodegradation problems [3]. On the other hand, the use of nanocarriers, such as liposomes, provides an enhancement of the efficiency of foliar applications [4], which depend on fertilizer absorption and mobility. Both latter requirements often fail in traditional foliar fertilization, since they are highly dependent on relative humidity and temperature [5]. In this field, it is key to take into account the different vesicles in order to obtain a suitable system for 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 entry through the stomatal pore [6,7]. The use of nanocarriers from natural sources have been investigated over the last years. These types of nanosystems are biodegradable and different works have been showing promising results in using 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 vesicle and the delivery of Zn into protoplast was reported [8]. Furthermore, an increase in the penetrability through stomatal pores for B and Fe was shown when micronutrients were encapsulated in vesicles [6]. These facts can be enhanced with the use of surfactant to modify the vesicle surface and therefore, efforts have been made to study and screen different surfactants to add to membrane vesicles for use in the delivery of mineral nutrients. In medical areas, many advances have been reached in the modification of liposome surfaces, for example, with polyethylene-glycol (PEG), which conjugated to the surface, increases the targeting to specific tissues [9]. Nevertheless, in agriculture there are limited results in this aspect.

In this study, we sought to characterize physicochemically the 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 the parameters that reflect the functionality of the membrane vesicles. This research supposes a preliminary screening, which, in this sense, gives rise to deepen the characterization and find the optimal nano-system to use in foliar fertilization.

2. Materials and Methods

2.1. Materials

Inflorescences of *Brassica oleracea* L. var. botrytis 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 surfactants 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 λ_{ex} = 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 k_{exp} is the fitted exponential rate constant, V_0 is the initial mean vesicle volume, A_v is the mean vesicle surface area, V_w is the molar volume of water and C_{out} 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 $p < 0.05$ were chosen to determine significant differences between treatments. Small letters on top of bars point to the significant differences between treatments.

3. Results

3.1. Physico-Chemical Characterization

Physicochemical measurements were carried out with different populations of vesicles. A selection by size was made by filtration with the objective of providing suitable vesicles with different sizes depending on final application. Three vesicle populations were determined, one with an average hydrodynamic diameter of 400 nm without filtration, and the other two with lower average hydrodynamic diameters after filtration of 0.45 µm (355 nm) and of 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, the zeta potential values were also not modified with the filtrations and a negative value about −30 mV was stablished for membrane vesicles (Figure 1b).

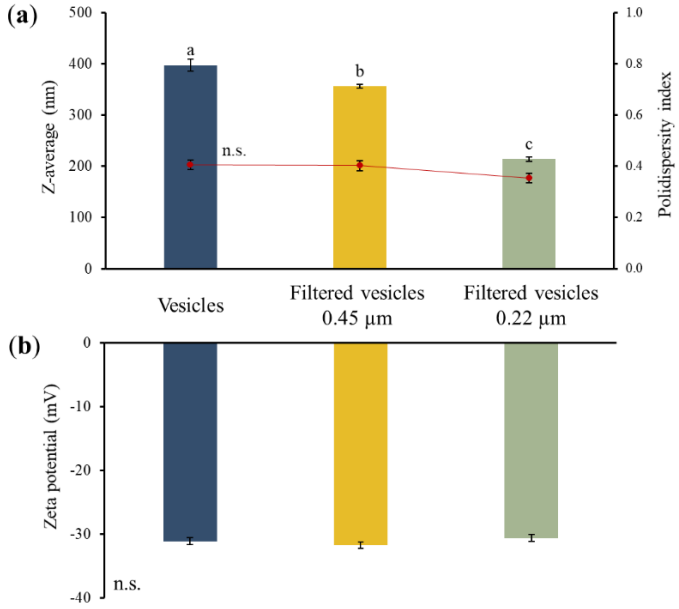

Figure 1. Physicochemical parameters membrane vesicles. (**a**) Z-average (nm) and polydispersity index and (**b**) zeta potential (mV) of vesicles, vesicles filtered by 0.45 µm and 0.22 µm. Data are means ± SE (n = 3).

3.2. Stability of Vesicles Functionality over Time

The functionality and stability of the 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).

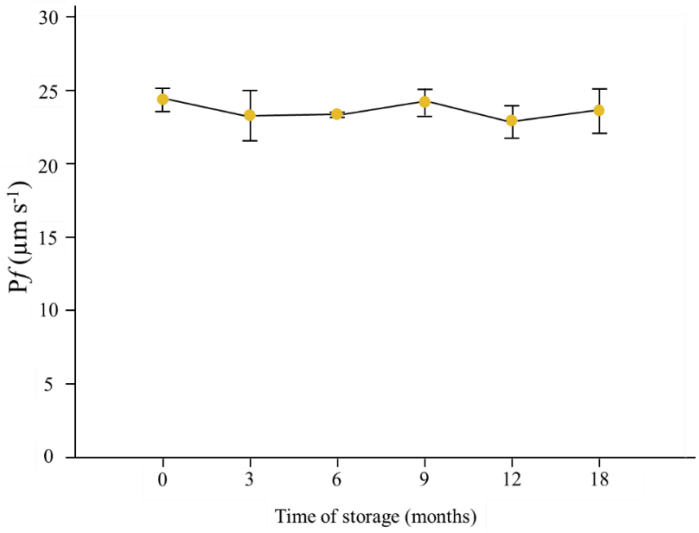

Figure 2. Membrane vesicle stability. Time course (months) of the osmotic water permeability (Pf) of membrane vesicles at 20 °C resuspended in potassium phosphate buffer and stabilized with polyalcohol. Data are means ± SE (n = 30).

3.3. Effect of Surfactant in Membrane Vesicle Functionallity

Once stability over time of the 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 displays *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. The results showed only the PMP modified the *Pf* of the 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, with a focus on nanocarriers [6,13]. However, it is still necessary to deepen the characterization and improvement of the nanosystems to reach higher efficiency. Proteoliposomes from natural sources such as *Brassica* plants have been shown to have potential use in different biotechnological applications, encapsulating bioactive compounds and delivering them in animal cells [14,15]. Furthermore, some studies have been carried out from an agricultural approach [6,8]. Based on this previous research, membrane vesicles from *Brassica oleracea* L. var. botrytis inflorescences were used to advance research to find the optimal nanosystems.

Different populations of membrane vesicles with different average sizes were obtained by filtration. Size is an important characteristic in nanobiotechnology and obtaining the optimal size could be key in the further applications. Sizes between 400 and 200 nm were obtained without modifying the homogeneity (PdI) and the charge (zeta potential) of the samples. These sizes are suitable for use in different applications [16], including foliar

application, in which 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 vesicle functionality was confirmed after storage time. The possibility of preserving the integrity of the nanosystems is essential for their use 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 improving the characteristics of nanosystems. The addition of surfactants could be interesting to increase the shelf-life or the capacity of the vesicles to have a specific target for delivery of the cargo (micronutrients, in this case) [17]. In this sense, different surfactants were tested in order to find out if the *Pf* of membrane vesicles was altered when surfactant was added; and only with PMP did the *Pf* value decrease. To determine the cause of the *Pf* decrease with PMP would need more investigation in relation to aquaporins, since these proteins are the transmembrane channels involved in the passage of water through membranes. Furthermore, 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 physicochemical characteristics and stability over time to be used in biotechnological applications, such as new technologies in agriculture. Furthermore, the 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, cargo targeting and efficiency, since some of surfactant tested did not alter the functionality of vesicles.

Author Contributions: Conceptualization, J.J.R. and M.C.; methodology, L.Y.-M.; validation, J.J.R. and M.C.; investigation, J.J.R., L.Y.-M. and M.C.; data curation, J.J.R. and L.Y.-M.; writing—original draft preparation, L.Y.-M.; writing—review and editing, J.J.R. and M.C.; visualization and supervision, J.J.R. and M.C.; project administration and funding acquisition, M.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministerio de Ciencia, Innovación y Universidades (AGL2016-80247-C2-1-R and RTC-2017-6544-2) and with a grant for L.Y.-M. (FPU-17/02261).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors thank Kernel Export, S.L. for providing the inflorescences of *Brassica oleracea* L. var botrytis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zhang, X.; Davidson, E.A.; Mauzerall, D.L.; Searchinger, T.D.; Dumas, P.; Shen, Y. Managing nitrogen for sustainable development. *Nature* **2015**, *528*, 51–59. [CrossRef] [PubMed]
2. Singh, R.P.; Handa, R.; Manchanda, G. Nanoparticles in sustainable agriculture: An emerging opportunity. *J. Control. Release* **2021**, *329*, 1234–1248. [CrossRef] [PubMed]
3. Niu, J.; Liu, C.; Huang, M.; Liu, K.; Yan, D. Effects of Foliar Fertilization: A Review of Current Status and Future Perspectives. *J. Soil Sci. Plant Nutr.* **2021**, *21*, 104–118. [CrossRef]
4. Karny, A.; Zinger, A.; Kajal, A.; Shainsky-Roitman, J.; Schroeder, A. Therapeutic nanoparticles penetrate leaves and deliver nutrients to agricultural crops. *Sci. Rep.* **2018**, *8*, 7589. [CrossRef] [PubMed]
5. Ramsey, R.J.L.; Stephenson, G.R.; Hall, J.C. A review of the effects of humidity, humectants, and surfactant composition on the absorption and efficacy of highly water-soluble herbicides. *Pestic. Biochem. Physiol.* **2005**, *82*, 162–175. [CrossRef]
6. Rios, J.J.; Yepes-Molina, L.; Martinez-Alonso, A.; Carvajal, M. Nanobiofertilization as a novel technology for highly efficient foliar application of Fe and B in almond trees. *R. Soc. Open Sci.* **2020**, *7*, 200905. [CrossRef] [PubMed]

7. Fanourakis, D.; Giday, H.; Milla, R.; Pieruschka, R.; Kjaer, K.H.; Bolger, M.; Vasilevski, A.; Nunes-Nesi, A.; Fiorani, F.; Ottosen, C.O. Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. *Ann. Bot.* **2015**, *115*, 555–565. [CrossRef] [PubMed]
8. Rios, J.J.; Garcia-Ibañez, P.; Carvajal, M. The use of biovesicles to improve the efficiency of Zn foliar fertilization. *Colloids Surf. B Biointerfaces* **2019**, *173*, 899–905. [CrossRef] [PubMed]
9. Allahou, L.W.; Madani, S.Y.; Seifalian, A. Investigating the Application of Liposomes as Drug Delivery Systems for the Diagnosis and Treatment of Cancer. *Int. J. Biomater.* **2021**, *2021*, 3041969. [CrossRef] [PubMed]
10. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
11. Karsch-Bluman, A.; Avraham, S.; Assayag, M.; Schwob, O.; Benny, O. Encapsulated carbenoxolone reduces lung metastases. *Cancers* **2019**, *11*, 1383. [CrossRef] [PubMed]
12. Maurel, C.; Tacnet, F.; Güclü, J.; Guern, J.; Ripoche, P. Purified vesicles of tobacco cell vacuolar and plasma membranes exhibit dramatically different water permeability and water channel activity. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7103–7108. [CrossRef] [PubMed]
13. Eichert, T.; Kurtz, A.; Steiner, U.; Goldbach, H.E. Size exclusion limits and lateral heterogeneity of the stomatal foliar uptake pathway for aqueous solutes and water-suspended nanoparticles. *Physiol. Plant.* **2008**, *134*, 151–160. [CrossRef] [PubMed]
14. Yepes-Molina, L.; Martínez-Ballesta, M.C.; Carvajal, M. Plant plasma membrane vesicles interaction with keratinocytes reveals their potential as carriers. *J. Adv. Res.* **2020**, *23*, 101–111. [CrossRef] [PubMed]
15. Yepes-Molina, L.; Hernández, J.A.; Carvajal, M. Nanoencapsulation of Pomegranate Extract to Increase Stability and Potential Dermatological Protection. *Pharmaceutics* **2021**, *13*, 271. [CrossRef] [PubMed]
16. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **2018**, *10*, 57. [CrossRef] [PubMed]
17. Milani, D.; Athiyah, U.; Hariyadi, D.M.; Pathak, Y. V Surface Modifications of Liposomes for Drug Targeting. In *Surface Modification of Nanoparticles for Targeted Drug Delivery*; Springer International Publishing: Cham, Switzerland, 2019; pp. 207–220.
18. Martínez-Ballesta, M.d.C.; García-Gomez, P.; Yepes-Molina, L.; Guarnizo, A.L.; Teruel, J.A.; Carvajal, M. Plasma membrane aquaporins mediates vesicle stability in broccoli. *PLoS ONE* **2018**, *13*, e0192422. [CrossRef] [PubMed]