Nanoparticles from different sources for nebulized drug delivery

ANNA ROMOLO, VID ŠUŠTAR, PROF. DR. VERONIKA KRALJ-IGLIČ
[1]University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics
Correspondence: prof. Veronika Kralj-Iglič, University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Zdravstvena 5, SI-1000 Ljubljana, Slovenia
e-mail: veronika.kralj-iglic@fe.uni-lj.si; tel:+386 41720766
webpage: http://www.lkbf.si/biophysics-and-biomechanics

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Abstract
Nanoparticles from different sources are being elaborated for drug delivery systems due to their ability to interact with cellular membranes and to convey their contents to the cells. Particles may be obtained from natural sources, can be formed in laboratory from prescribed ingredients or can be formed by combining natural and synthetic ingredients. In this work we present examples of natural nanoparticles from the blood and from microalgae Phaeodactylum tricornutum, and liposomes.

Nanodelci iz različnih virov za nebuliziran prenos učinkovin

Povzetek
Nanodelci iz različnih virov so predmet študija kot sistemi za prenos zdravilnih učinkovin, zaradi sposobnosti interakcije s celičnimi membranami in prenosa svojih sestavin v celice. Delci so lahko pridobljeni iz naravnih virov, lahko jih oblikujemo v laboratoriju iz predpisanih sestavin ali pa se tvorijo s kombinacijo naravnih in sintetičnih sestavin. V tem delu predstavimo primere nanodelcev: liposome, nanodelce iz krvi in nanodelce iz kulture in izolata mikroalge Phaeodactylum tricornutum.

1. INTRODUCTION

To reach the optimal therapeutic activity, the drug should be delivered to the target site at a proper amount and rate. Inhaled drug delivery is an efficient and minimally invasive method of drug delivery with some advantages with respect to intravenous or oral administration: less side effects due to direct access, optimized therapeutic index of the drug, enhanced retention of the drug at the target site, decrease of the frequency of administration and more uniform concentration of the drug with respect to time (Zaru et al., 2007; Agarwaal, 2022).

Lipid membrane-enclosed vesicles are especially suited for delivery to cells, for their structural resemblance to cellular membranes. Likewise, in the vesicles, the membrane divides the content from the aqueous medium. Due to hydrophobic interaction, lipid molecules configure in the form of bilayer to minimize the contact between the hydrophobic tails with water molecules and provide also the surrounding for other hydrophobic constitutive molecules or their parts. Membrane-enclosed vesicles and micelles are likely formed. Externally added hydrophobic compounds preferentially distribute within the lipid bilayer, hydrophilic compounds distribute inside or outside the vesicles and amphiphilic compounds distribute at the border between the membrane and the surroundings. Also, they orient in such way as to allow hydrophobic part to be shielded from water and hydrophilic part to be in contact with water. Shape, size and composition of these particles are subjected to minimization of the free energy of the whole system which is described as self-assembly, therefore we call such particles colloidal vesicles (Kralj-Iglič et al., 2020; 2022a). Examples of colloidal vesicles are liposomes and extracellular vesicles.

Liposomes can form spontaneously by mixing appropriate amounts of phospholipids and water (Lukawski et al., 2020). Phospholipids can be of natural, partially synthetic, or synthetic origin. Phospholipids favor the formation of lamellae, due to their two-tailed structure which requires a proper tail-to head lateral extension ratio to favor bilayers with small curvature. Different methods for preparing liposomes were developed, which yield particles with different sizes: small unilamellar vesicles (SUVs) below 100 nm, large unilamellar vesicles (LUVs) between 100 nm and 1 μm, giant unilamellar vesicles (GUVs) larger than 1 μm and multilamellar vesicles (MLVs) with onion-like structure; with low volume-to-surface area ratio, SUVs are appropriate for loading hydrophobic substances to the membrane while LUVs with higher volume-to surface area ratio are appropriate for loading hydrophilic substances into the vesicle lumen (Rudokas et al., 2016). The properties of the different liposomal classes determine their applicability. With distinct properties like biocompatibility and biodegradability and due to their nano-size, liposomes have potential applications in delivery of antibiotics, anti-fungal, anticancer drugs and genetic medicines; a sustained release of drug compounds from the liposomal structures was achieved most efficiently with a MLV structure, which is advantageous as the compound must cross several lipid bilayers before release (Schmehl et al., 2016). Liposomes can entrap anticancer drugs and localise their action in the lung following pulmonary delivery. The safety of inhaled liposomes incorporating anticancer drugs depends on the anticancer agent used and the amount of drug delivered to the target cancer in the lung. Liposome preparations are a subject of extensive research, however, there remain many challenges like pharmaceutical manufacturing, quality assurance, cost, and in some cases, insufficient efficiency (Ibrahim et al., 2022).

To overcome the latter, natural drug carriers are being considered (Popowski et al., 2022). Cells naturally shed into their surroundings small cellular particles (SCPs). Among them are also membrane-enclosed small cellular vesicles that are formed in a process in which membrane plays a key role (Kralj-Iglič et al., 2022a). After being released into the cell surroundings, they may move more or less freely and with body fluid transport reach the distant cells. It is indicated that they play an important role in living systems as they present an intercellular communication system. SCPs including microexovesicles, exosomes, enveloped viruses, and cellular membrane endovesicles are naturally optimized for mRNA encapsulation and cellular delivery. Being cellular fragments, they include constituents of the mother cell. SCPs have already been used as therapeutic agents in various disease applications (see for example Vozel et al., 2021). SCPs should be harvested from their natural environment e.g. by differential centrifugation optionally followed by using for example sucrose or iodixanol gradient, by ultrafiltration,

by flow field-flow fractionation, by dialysis, by size exclusion chromatography, by microchip-based techniques, by precipitation and by immunoaffinity (Kralj-Iglič et al., 2022a). Recently, a number of commercial kits are made available such as ExoQuick (System Biosciences), Total Exosome Isolation kit (TEI, Invitrogen); qEV (Izon); Millipore; exoEasy (Qiagen)(Kralj-Iglič et al., 2022a). However, the contents of the isolates depend on the methods of harvesting (Božič et al., 2020), the samples are heterogeneous in composition and size and characterization methods are unable to indicate the origin of SCPs.
Due to the above, further research is needed to study and optimize drug delivery systems. In this work we give the data on morphology of three types of potential drug carriers: liposomes composed from lecithin, glycerol and water, colloidal vesicles from blood and SCPs from microalgae Phaeodactylum tricornutum.

2. METHODS

Preparation of liposomes
Edible soya Lecithin was from Fiorentini, Torino, Italy. Liposomes were prepared by mixing appropriate proportions of liophylized soya lecithin granules with ultraclean water and glycerol at room temperature (Lukawski et al., 2020, Romolo et al., 2022). A sample contained 25 weight % of soya granules, 50 % of water and 25% of glycerol. Soyabean lecithin granules were put into the falcon tubes, water was added and the suspension was left at room temperature for 1 hour. Glycerol was added and the samples were mixed by pipetting with Pasteur pipette.

Blood sampling
20 mL of blood was collected in 2.7 mL tubes containing 270 μL trisodium citrate at a concentration of 0.109 mol/L. Evacuated tubes (BD Vacutainers, Becton Dickinson, CA) were used in all the experiments. A 21-gauge needle (length 70 mm, inner radius 0.4 mm, Microlance, Becton Dickinson, NJ) was used for blood sampling in all the experiments. A 21 gauge needle (Tik d.o.o., Kobarid, Slovenia) was used. The covers of the vacutubes were removed prior to sampling and the blood was allowed to drop freely into the tube. Variation in the acquired volumes did not exceed 15%. The tubes were incubated in a rotating centrifuge at room temperature and kept in a water bath during handling of the samples. The samples were left in the water bath for several minutes to attain the desired temperature before centrifugation.

Isolation of nanoparticles
Centrifugation of the samples started within 20 minutes after acquisition of the sample. In order to separate the cells from plasma, the samples were centrifuged at 1550 × g for 20 minutes in a Centric 400/R centrifuge (Tehtnica Železniki, Železniki, Slovenia). Plasma was removed, mixed, aliquoted in 250 μL samples in Eppendorf tubes and centrifuged at 17570 × g for 30 minutes in a Centric 200/R centrifuge (Tehtnica Železniki). The supernatants (225 μL) were discarded and the pellets (25 μL) resuspended in 225 μL citrated phosphate-buffered saline samples. The resuspended samples were centrifuged again at 17570× g for 30 minutes. The supernatants (225 μL) were discarded and the pellets (25 μL) were collected and resuspended in 75 μL of citrated phosphate-buffered saline.

Cultivation of the algae
Cultures of Tetraselmis chuii CCAP 66/21b, and Phaeodactylum tricornutum CCAP 1052/1A from the Culture Collection of Algae and Protozoa (CCAP) of SAMS (Oban, Scotland) were grown in artificial seawater (Reef Crystals, Aquarium Systems, France). 22 g of salt was dissolved in one litre of distilled water, sterile filtered (0.2-micron cellulose filters, ref. 11107-47-CAN, Sartorius Stedim Biotech GmbH, Germany), autoclaved, and supplemented with Guillard's (F/2) Marine Water Enrichment Solution (ref. G0154, Sigma Aldrich, USA)18. Cultures were grown in a respirometer (Echo, Slovenia) in 0.5-L borosilicate bottles, at 20 °C and 20 % illumination with a 14-hour light / 10-hour dark cycle, with aeration of 0.2 L/min.

Isolation of SCPs from microalgae samples

SCPs were isolated by differential centrifugation, using a protocol widely used for the isolation of small extracellular vesicles. The cells were removed by low-speed centrifugation (300 g, 10 min, 4°C, centrifuge Centric 260R with rotor RA 6/50 (Domel, Slovenia)), using 50 mL conical centrifuge tubes (ref. S.078.02.008.050, Isolab Laborgeräte GmbH, Germany); and 2000 g, 10 min, 4°C (Centric 400R centrifuge with rotor RS4/100 (Domel, Slovenia)), using 15 mL conical centrifuge tubes (ref. S.078.02.001.050, Isolab La-borgeräte GmbH, Germany). Each step was repeated twice. Then, the cell-depleted medium was centrifuged twice at 10 000 g and 4°C for 30 min (Beckman L8-70M ultracentrifuge, rotor SW55Ti (Beckman Coulter, USA)), using thin-wall polypropylene centrifuge tubes (ref. 326819, Beckman Coulter, USA) to remove larger cell debris. Finally, SCPs were pelleted by ultracentrifugation at 118 000 g and 4°C for 70 min in the same type of ultra-centrifuge and ultracentrifuge tubes. The pellet was resuspended in 50 µL of marine water.

Electron microscopy

Plasma and plasma SCPs were imaged by scanning electron microscopy (SEM). SCPs were suspension-fixed in 1% glutaraldehyde dissolved in phosphate-buffered saline/citrate buffer for 60 minutes at 22°C, post-fixed for 60 minutes at 22°C in 1% OsO_4 dissolved in 0.9% NaCl, dehydrated in a graded series of acetone/water (50%–100%, v/v) and critical point dried. The samples were gold-sputtered and examined using a LEO Gemini 1530 (LEO, Oberkochen, Germany) scanning electron microscope. Drying may have caused shrinking of objects up to 20%, the effect being more pronounced for larger objects (cells).

Microalgae and microalgae SCPs were imaged by SEM and cryogenic electron microscopy (cryo-TEM) as described in (Romolo et al., 2022) and (Kralj-Iglič et al., 2022b).

3. RESULTS

Figure 1 shows liposomes that were formed by mixing liophilized granules of lecithin, water and glycerol. We can see an enormous number of droplets and colloid vesicles of different sizes. Some of these particles are huge (tens of micrometers), however the smaller ones seem more numerous. At the 10 times smaller scale (Panel D) a similar texture can be recognized. The resolution of the light microscope limits approach to even smaller scale. The sample imaged consisted of a microliter droplet of liposome preparation while 30 milliliters of the preparation were prepared by mixing equal weight proportions of lecithin, watr and glycerol.

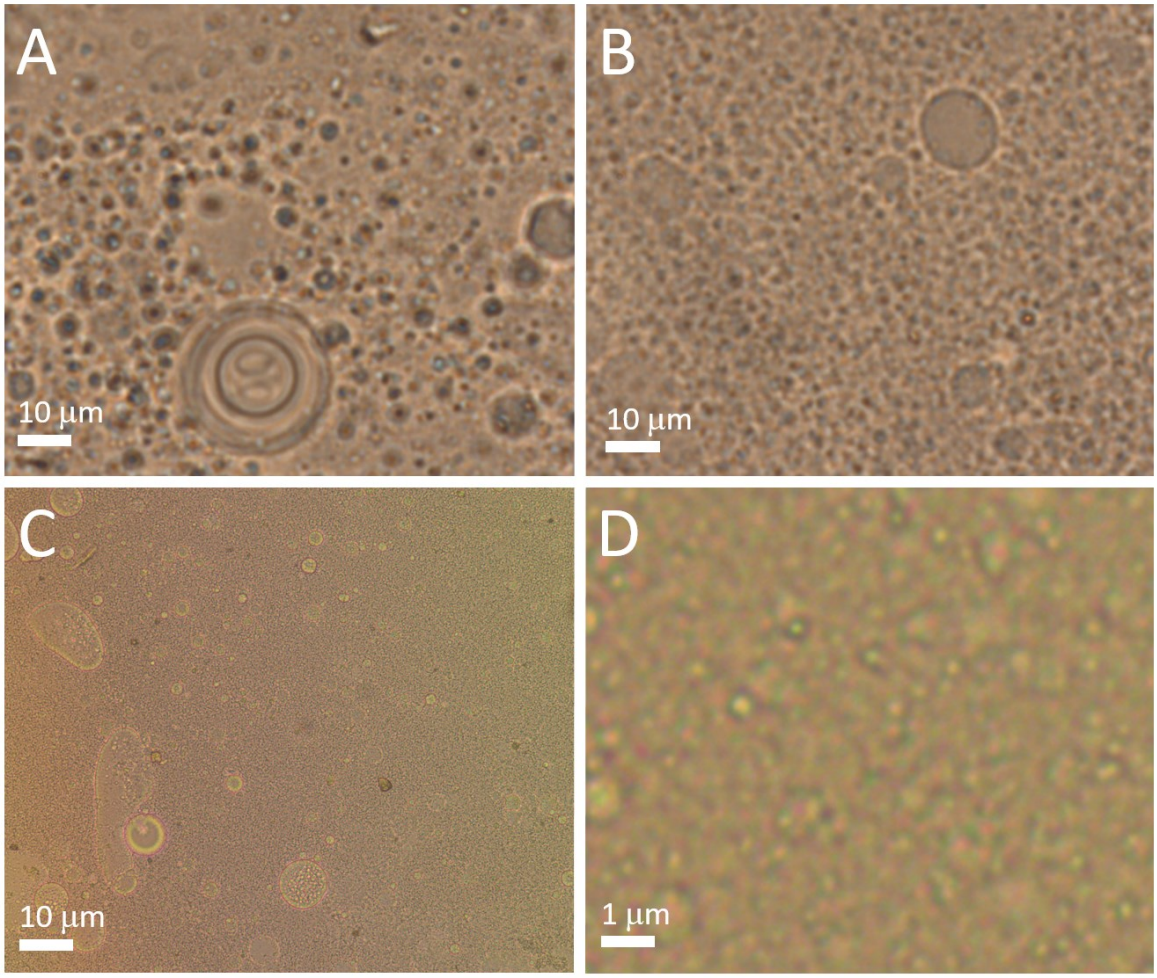

Figure 1. Liposomes formed by mixing liophilized granules of lecithin with water and glycerol as observed by the light microscope.

Figure 2A shows imaged plasma. Two residual erythrocytes are observed in the center of the image, while around them there are numerous platelets. Platelets are activated as evidenced by their shape that deviates from the thin disk-like shape. Tubular protrusions can be observed in some platelets. Also, nano-sized fragments are visible, in particular those deposited on the surface of the upper erythrocyte. Figure 2B shows budding of the erythrocytes. Small round buds are formed at the tips of the echinocyte spicules. These buds eventually pinch off and become free SCPs with diameters in the 50-200 nm range. Previous results showed that these SCPs are colloidal membrane-bound vesicles filled with haemoglobin but devoid of membrane skeleton (Kralj-Iglic et al., 2020). Figures 2C and 2D show isolates from plasma. Isolates are rich with SCPs of smooth shapes characteristic for colloidal vesicles (Kralj-Iglič et al., 2022). The most apparent SCPs have effective diameters of 200-500 nm, however, there were also smaller SCPs in the isolate. Isolate rich with SCPs was prepared from about 20 ml of blood. The whole isolate was mounted on the stand for imaging.

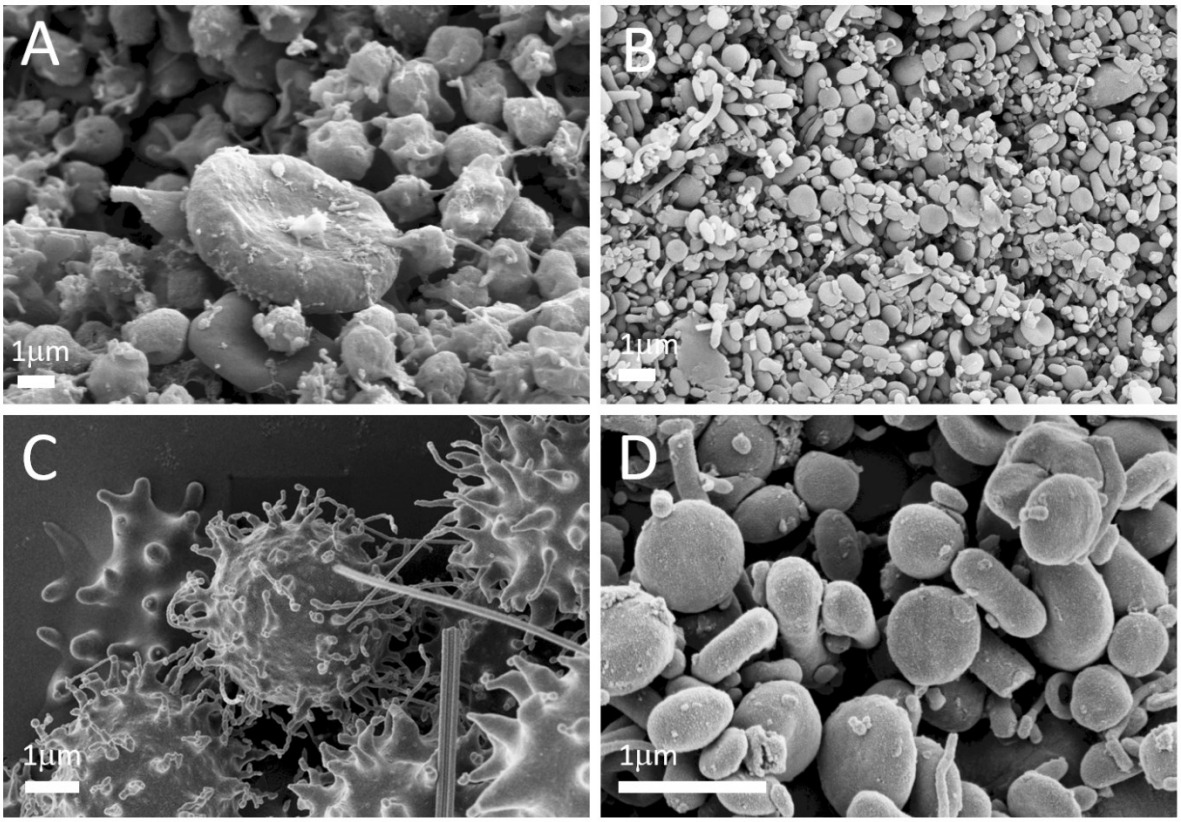

Figure 2. Scanning electron microscope images of platelet-rich plasma (A), budding erythrocytes (B), isolate from plasma (C) colloid vesicles isolated from plasma.

Figure 3A shows imaged microalgae Phaeodactylum tricornutum. Numerous microalgae can be seen. Figure 4B shows budding of the microalgae surface. It seems that the material at the surface forms wrinkles that in some places exhibit small globular buds of diameters less than 100 nm. Cryo-TEM image (Figure 4C) reveals the composition of the isolate; different types of SCPs, and ultrastructure of SCPs. Electron-dense globular SCPs (black arrows), vesicles (white arrows) and clusters (dotted white arrows) can be seen. As all these particle types can be of the same size, they cannot be distinguished in the SEM images (Figures 2B and 2D). Figure 2D shows isolated SCPs from culture of Phaeodactylum tricornutum. We can see that the particles are homogeneous in size and shape and that the size corresponds to the size of particles observed in Figure 2C. Numerous thin filaments observed in Figure 2C may impose obstacles in sedimentation of SCPs and also present difficulties in analysing the size by methods that utilize the Stokes – Einstein equation for determination of hydrodynamic radius of the particles from records of Brownian motion (Romolo et al., 2022). Isolates from Phaeodactylum tricornutum starts from 50 mL of culture and yields minute amount of the isolate that is not visible by eye.

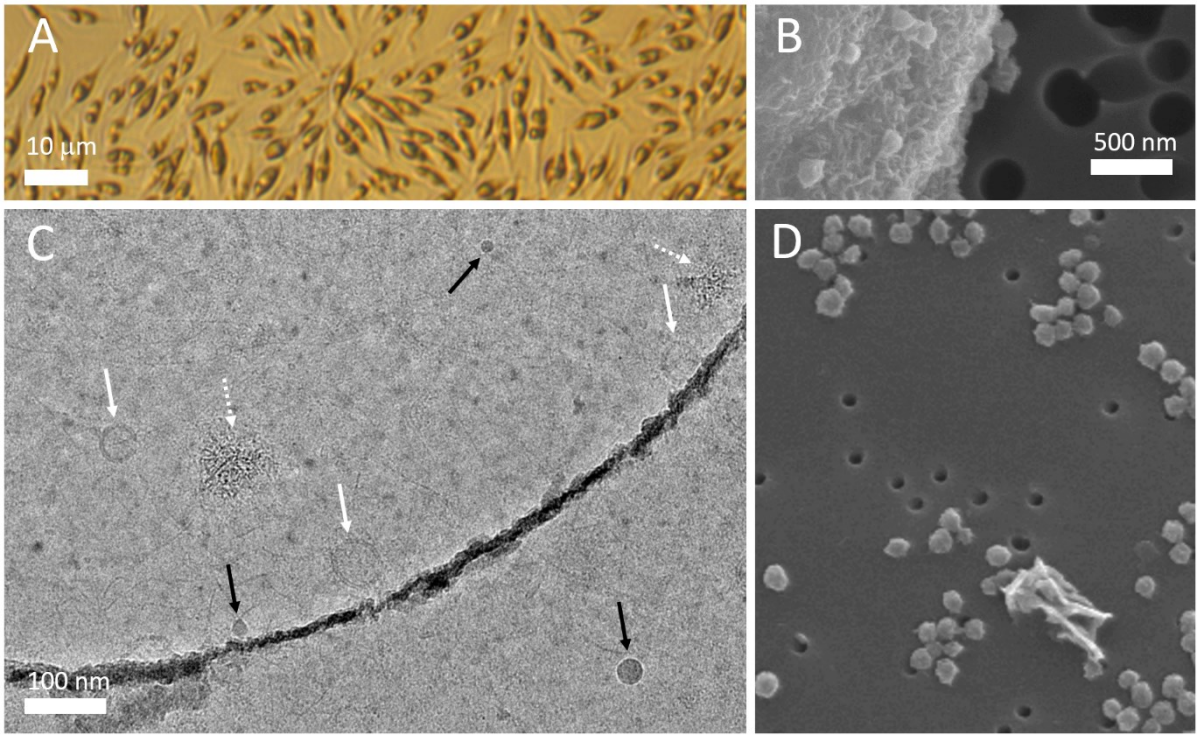

Figure 3. Microalgae Phaeodactylum tricornutum (A), budding of the cell surface (B), small cellular particles in the isolate (C) and in the culture (D). A: light microscopy, B and D scanning electron microscopy, C: cryo-electron microscopy. B-D from (Kralj-Iglič et al., 2022b).

4. DISCUSSION

We present three types of nanoparticles considered as potential platforms for drug delivery. Liposome formation (mixing appropriate amounts of lipid, glycerol and water) is a simple and inexpensive method suitable for large-scale production. The size of liposomes vary over three orders of magnitude, however, smaller liposomes are the most numerous ones as evidenced by their average hydrodynamic radius (Kralj-Iglič et al., 2022b). Furthermore, the liposomes can be stable for 6 months at room temperature (Romolo et al., 2022). Also erythrocyte-derived colloidal vesicles (Bozic eet al., 2021; Jozelj et al., 2022) have exhibited stability over 6 month period (Bozic et al., 2021). SCPs from blood plasma are natural particles. It is advantageous that in healthy subjects, they can be prepared from the subject's own body fluid. On the other hand, their use is contra-indicated in patients with cancer, inflammation or infection. Plasma contains a wide variety of substances and particles and its composition depends on subject's prandial state and activity. It remains to be investigated how the subject should be prepared for the optimal plasma auto-donation. Also, it is yet not completely clear how saving of plasma affects its quality (content of bioactive molecules, SCPs and blood cells). Microalgae have recently been considered as a potential source of SCPs for drug delivery (Picciotto et al. 2021, Adamo et al., 2021). We have observed particles sized between 100 and 200 nm in microalgae culture and in isolates from conditioned media of microalgae Phaeodactylum tricornutum (Figure 3), in accordance with measurements by dynamic light scattering (Bozic et al., 2022, Romolo et al., 2022). However, SCP yield was rather low, in particular when compared with the yield of liposomes or SCPs isolated from plasma. Furthermore, microalgae grow in co-culture with other microorganisms, e.g. bacteria (Škufca et al., 2022), which presents an obstacle in their consideration for internal administration.

Nebulization may involve forces that change the partciles' integrity, size and shape and therefore loss of the drug (Elhissi, 2017). Therefore, a sufficient stability of the aerosolized nanoparticle is necessary for pulmonary application. It would be appropriate to design liposomes by carefully selecting the drug lipid ratio, lipid composition, etc. to achieve their optimal lifetime and drug release rate. Processes of

liposome formation as well as dilution for assessment influence the samples (Elhissi et al., 2006, Lehofer et al., 2014), i.e., the shape, size and number density of liposomes. Likewise processes of isolation influence the identity, number density and morphology of SCPs (Božič et al., 2020).

5. CONCLUSIONS

Liposomes and plasma-derived nanoparticles are membrane-enclosed vesicles. Isolates from microalgae Phaeodactylum tricornutum contain nanovesicles but also other types of nanoparticles. Yield of liposomes was very high, yield of plasma-derived vesicles was high and yield of SCPs isolated from microalgae Phaeodactylum tricornutum by ultracentrifugation was low.

6. ACKNOWLEDGEMENTS

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7. REFERENCES

1. *Adamo G, Fierli D, Romancino DP, Picciotto S, Barone ME, Aranyos A, Božič D, Morsbach S, Raccosta S, Stanly C, Paganini C, Gai M, Cusimano A, Martorana V, Noto R, Carrotta R, Librizzi F, Randazzo L, Parkes R, Capasso Palmiero U, Rao E, Paterna A, Santonicola P, Iglič A, Corcuera L, Kisslinger A, Di Schiavi E, Liguori GL, Landfester K, Kralj-Iglič V, Arosio P, Pocsfalvi G, Touzet N, Manno M, Bongiovanni A. Nanoalgosomes: Introducing extracellular vesicles produced by microalgae. J Extracell Vesicles. 2021, 10: e12081. DOI: 10.1002/jev2.12081*
2. *Agarwal K, Liposome assisted drug delivery-an updated review, Indian J Pharm Sci 2022, 84(4): 797-811. DOI: 10.36468/pharmaceutical-sciences*
3. *Božič D, Hočevar M, Kononenko V, Jeran M, Štibler U, Fiume I, Pajnič M, Pađen L, Kogej K, Drobne D, Iglič A, Pocsfalvi G, Kralj-Iglič V. Pursuing mechanisms of extracellular vesicle formation. Effects of sample processing. Advances in Biomembranes and Lipid Self-Assembly (Bongiovanni A, Pocsfalvi G, Manno M, Kralj-Iglič V, Eds). 2020, 32: 113-155*
4. *Božič D, Hočevar M, Kisovec M, Pajnič M, Pađen L, Jeran M, Zavec Bedina A, Podobnik M, Kogej K, Iglič A, Kralj-Iglič V. Stability of erythrocyte-derived nanovesicles assessed by light scattering and electron microscopy. Int J Mol Sci. 2021, 22-23:12772. DOI: 10.3390/ijms222312772*
5. *Božič D, Hočevar M, Jeran M, Kisovec M, Bedina Zavec A, Romolo A, Škufca D, Podobnik M, Kogej K, Iglič A, Touzet N, Manno M, Pocsfalvi G, Bongiovanni A, Kralj-Iglic V. Ultrastructure and stability of cellular nanoparticles isolated from Phaeodactylum tricornutum and Dunaliella tertiolecta conditioned media Open Res Europe 2022, 2:121*
6. *Elhissi A. liposomes for pulmonary drug delivery: the role of formulation and inhalation device design. Curr Pharm Des. 2017, 23: 362-372. DOI: 10.2174/1381612823666161116114732*
7. *Ibrahim JP, Haque S, Bischof RJ, Whittaker AK, Whittaker MR, Kaminskas LM. Liposomes are poorly absorbed via lung lymph after inhaled administration in sheep. Front Pharmacol. 2022, 13: 880448. DOI: 10.3389/fphar.2022.880448*
8. *Jozelj M, Košir T, Božič D, Hočevar M, Pajnič M, Iglič A, Jeran M, Kralj-Iglič V. Using a physical approach to study morphological properties erythrocyte extracellular vesicles. Proceedings of Socratic Lectures. 2022, 7: 111-115. DOI: 10.55295/PSL.2022.D16*
9. *Kralj-Iglič V, Pocsfalvi G, Mesarec L, Šuštar V, Hägerstrand H, Iglič A. Minimizing isotropic and deviatoric membrane energy – An unifying formation mechanism of different cellular membrane nanovesicle types. PLoS ONE. 2020; 15: e0244796. DOI: 10.1371/journal.pone.0244796*
10. *Kralj-Iglič V, Pocsfalvi G, Iglič, A. morphology and formation mechanisms of cellular vesicles harvested from blood. extracellular vesicles - role in diseases, pathogenesis and therapy. Edited by Manash Paul, IntechOpen, January 25th, 2022a*
11. *Kralj-Iglič V, Bedina Zavec A, Božič D, Hočevar M, Iglič A, Jeran M, Kisovec M, Podobnik M, Romolo A, Škufca D. Scanning electron microscope images of Dunaliela tertiolecta and Phaeodactylum*

tricornutum cultures and scanning electron microscope images and cryogenic electron microscope images of isolated small cellular particles from respective conditioned media. Zenodo. 2022b. DOI: 10.5281/zenodo.6908895

12. *Lehofer B, Bloder F, Jain PP, Marsh LM, Leitinger G, Olschewski H, Leber R, Olschewski A, Prassl R, Impact of atomization technique on the stability and transport efficiency of nebulized liposomes harboring different surface characteristics, Eur J Pharm Biopharm, 2014, 88: 1076-1085. DOI: 10.1016/j.ejpb.2014.10.009*
13. *Łukawski M, Dałek P, Borowik T, Foryś A, Langner M, Witkiewicz W, Przybyło M. New oral liposomal vitamin C formulation: properties and bioavailability. J Liposome Res. 2020, 30: 227-234. DOI: 10.1080/08982104.2019.1630642*
14. *Picciotto S, Barone ME, Fierli D, Aranyos A, Adamo G, Božič D, Romancino DP, Stanly C, Parkes R, Morsbach S, Raccosta S, Paganini C, Cusimano A, Martorana V, Noto R, Carrotta R, Librizzi F, Palmiero UC, Santonicola P, Iglič A, Gai M, Corcuera L, Kisslinger A, Di Schiavi E, Landfester K, Liguori GL, Kralj-Iglič V, Arosio P, Pocsfalvi G, Manno M, Touzet N, Bongiovanni A. Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds. Biomater Sci. 2021, 9: 2917-2930. DOI: 10.1039/d0bm01696a*
15. *Popowski KD, López de Juan Abad B, George A, Silkstone D, Belcher E, Chung J, Ghodsi A, Lutz H, Davenport J, Flanagan M, Piedrahita J, Dinh PUC, Cheng K, Inhalable exosomes outperform liposomes as mRNA and protein drug carriers to the lung, Extracellular Vesicle, 2022, 1: 100002. https://doi.org/10.1016/j.vesic.2022.100002.*
16. *Romolo A, Jan Z, Bedina Zavec A, Kisovec M, Arrigler V, Podobnik M, Iglič A, Pocsfalvi G, Kogej K, Kralj-Iglic V. Assessment of small cellular particles from four different natural sources and liposomes by interferometric light microscopy. Int J Mol Sci. 2022, 23, submitted*
17. *Rudokas M, Najlah M, Alhnan MA, Elhissi A. Liposome Delivery Systems for Inhalation: A Critical Review Highlighting Formulation Issues and Anticancer Applications. Med Princ Pract. 2016, 25 Suppl 2: 60-72. DOI: 10.1159/000445116*
18. *Schmehl T, Gessler T, Waschkowitz E, Nebulized liposomes for the pulmonary application of drug compounds https://patents.google.com/ patent/US8652512B2/en*
19. *Škufca D, Božič D, Hočevar M, Jeran M, Bedina Zavec A, Kisovec M, Podobnik M, Matos T, Tomazin R, Iglič A, Griessler Bulc T, Heath E, Kralj-Iglič V. Interaction between Microalgae P. tricornutum and Bacteria Thalassospira sp. for Removal of Bisphenols from Conditioned Media. Int J Mol Sci 2022, 23: 8447. DOI: 10.3390/ijms23158447*
20. *Vozel D, Božič D, Jeran M, Jan Z, Pajnič M, Pađen M, Steiner N, Kralj-Iglič V, Battelino S. Autologous platelet-and extracellular vesicle-rich plasma is an effective treatment modality for chronic postoperative temporal bone cavity inflammation: Randomized controlled clinical trial. Front Bioeng Biotechnol. 2021, 9: 677541. DOI: 10.3389/fbioe.2021.677541*
21. *Zaru M, Mourtas S, Klepetsanis P, Fadda AM, Antimisiaris SG. Liposomes for drug delivery to the lungs by nebulization. Eur J Pharm Biopharm. 2007, 67: 655-66.DOI: 10.1016/j.ejpb.2007.04.005*