UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Lipid-Protein Nanovesicles of Membrane from *Brassica Oleracea* L. Var. *italica* as Carriers of Bioactive Compounds

Nanovesículas Lípido-Proteicas de Membrana de *Brassica Oleracea* L. Var. *italica* como Transportadores de Compuestos Bioactivos

D.ª Lucía Yepes Molina

2022

The present doctoral thesis entitles "*Lipid-protein nanovesicles of membrane from Brassica oleracea* L. *var. italica as carriers of bioactive compounds*" is presented as a compendium of the following research published articles with the agreement of the doctoral thesis directors and the responsible of Academic Committee for the doctoral program in Molecular Biology and Biotechnology:

- **Yepes-Molina, L**., Martinez-Ballesta, M. C., & Carvajal, M. Plant plasma membrane vesicles interaction with keratinocytes reveals their potential as carriers. (2020). *Journal of advanced research*, 23, 101-111.

- **Yepes-Molina, L**., & Carvajal, M. Nanoencapsulation of sulforaphane in broccoli membrane vesicles and their *in vitro* antiproliferative activity. (2021). *Pharmaceutical Biology*, 59(1), 1490-1504.

- **Yepes-Molina, L**., Perez-Jimenez, M.I., Martinez-Esparza, M., Teruel, J.A., Ruiz-Alcaraz, A.J., Garcia-Peñarubia, P., & Carvajal, M. Membrane vesicles for nanoencapsulate sulforaphane increased their anti-inflammatory role on *in vitro* human macrophage model. (2022). *International Journal of Molecular Science*, 23(4), 1940.

In addition to publication included in the compendium, additional publications have derived from this thesis:

Scientific journal:

- Martínez-Ballesta, M. D. C., García-Gómez, P., **Yepes-Molina, L**., Guarnizo, A. L., Teruel, J. A., & Carvajal, M. Plasma membrane aquaporins mediates vesicle stability in broccoli. (2018). *PLoS One*, 13(2), e0192422.

- Martinez-Ballesta, M.C., García-Ibáñez, P., **Yepes-Molina, L.**, Rios, J.J., & Carvajal, M. (2018). The expanding role of vesicles containing aquaporins. *Cells*, 7(10), 179.

- **Yepes-Molina, L.**, Chelbi, N., Vivo, J.M., Franco, M., Agudelo, A., Carvajal, M., & Martinez-Ballesta, M.C. Analysis of physiological traits in the response of Chenopodiaceae, Amaranthaceae, and Brassicaceae plants to salinity stress. (2018). *Plant Physiology and Biochemistry*, 132, 145-155.

- **Yepes-Molina, L.**, Carvajal, M., & Martinez-Ballesta, M.C. Detergent resistant membrane domains in broccoli plasma membrane associated to the response to salinity stress. (2020). *International Journal of Molecular Sciences*, 21(20), 7694.

- 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, (2020). *Royal Society Open Science*, 7(11), 200905.

- **Yepes-Molina, L.**, Bárzana, G., & Carvajal, M. Controversial regulation of gene expression and protein transduction of aquaporins under drought and salinity stress. (2020). *Plants*, 9(12), 1662.

- García-Ibáñez, P., **Yepes-Molina, L.**, Ruiz-Alcaraz, A.J., Martínez-Esparza, M., Moreno, D.A., Carvajal, M., & García-Peñarrubia, Pilar. Brassica bioactives could ameliorate the chronic inflammatory condition of endometriosis, (2020). *International Journal of Molecular Sciences*, 21(24), 9397.

- **Yepes-Molina, L.,** Hernández, J.A., & Carvajal, M. Nanoencapsulation of pomegranate extract to increase stability and potential dermatological protection. (2021). *Pharmaceutics*, 13(2), 271.

- Barzana, G., Rios, J.J., López-Zaplana, A., Nicolás-Espinosa, J., **Yepes-Molina, L.,** García-Ibáñez, P., & Carvajal, M. Interrelations of nutrient and water transporters in plants under abiotic stress. (2021). *Physiologia Plantarum*, 171(4), 595-619.

- Nicolas-Espinosa, J., **Yepes-Molina, L.,** & Carvajal, M. Bioactive peptides from broccoli stems strongly enhance regenerative keratinocytes by stimulating controlled proliferation. (2022). *Pharmaceutical biology*, 60(1), 235-246.

Contribution to conferences and congress:

- **Yepes-Molina, L.,** Moreno, D.A., Dominguez-Perles, R., Martinez-Ballesta, M.C. & Carvajal, M. Poster: Nanoencapsulated glucosinolates for nutraceutical applications. (2018). *IV International congress food technology, quality and safety*. Novi Sad (Serbia), 23/10/2018 – 25/10/2018.

- **Yepes-Molina, L.**, Martinez-Ballesta, M.C., Guarnizo-Serrudo, A.L., Teruel, J.A. & Carvajal, M. Poster and flash talk: Integrity of broccoli plasma membrane to *in vitro* thermal applications. (2019). *18th International Workshop on Plant Membrane Biology.* Glasgow (Scotland), 7/7/2019 – 12/7/2019.

- **Yepes-Molina, L.**, Martinez-Ballesta, M.C. & Carvajal, M. Oral communication: Transdermal delivery of nanocarrier vesicles obtained from broccoli. (2021). *V Jornadas Doctorales Universidad de Murcia.* Murcia (Spain), 21/6/2021 – 24/6/2021.

- **Yepes-Molina, L.**, Rios, J.J. & Carvajal, M. Proceeding paper: Physicochemical characterization and effect of additives of membrane vesicles from *Brassica oleracea* L. to be used in nanofertilization. (2021). *The 2nd International Electronic Conference on Plant Sciences.* Online, 1/12/2021 – 15/12/2021.
- Nicolás-Espinosa, J., **Yepes-Molina, L.,** & Carvajal, M. Poster: Plasma membrane aquaporins as early molecular markers of boron stress in *Brassica oleracea*. var. *italica* leaves. (2022). *XVI Meeting of plant molecular biology*. Seville (Spain), 14/9/2022 – 14/9/2022.

The author of this thesis memory has jointed of PhD student grant programme “Formación del Profesorado Universitario (FPU)” (FPU17/02261) from the Ministry of Universities (Spain).

The research described in this thesis was financially supported by the projects:

- AGL2016-80247-C2-1-R. Development of nanotechnologies for encapsulation of pomegranate extracts with lipid-protein membrane vesicles for inclusion in nutricosmetics matrixes. Ministry of Science, Innovation and Universities (Spain).

- IDI-20190728. Functional differentiation of broccoli by-products as a basis for their cosmeceutical application. AGROPEYFI, S.L. - CDTI (Center for Industrial Technological Development).

A mi padre, la persona que me acompaña en cada arcoíris.

Agradecimientos

Todo el mundo te dice que hacer la tesis doctoral no es un camino fácil, y cuentas con ello cuando empiezas, pero lo que nadie dice es lo difícil que es escribir los agradecimientos y resumir 5 años en unas líneas. Aquí quiero reflejar que esta tesis doctoral es fruto del esfuerzo, pero también del cariño de todos los que me han acompañado.

En primer lugar, quiero agradecer a la Universidad de Murcia por ofrecerme una educación de calidad y multitud de enseñanzas no solo a nivel académico, sino también a nivel personal, desde que comencé el grado en Biotecnología hasta hoy que finalizo mi tesis doctoral. También quiero agradecer al Consejo Superior de Investigaciones Científicas (CSIC) y en concreto a todas las personas que forman el Centro de Edafología y Biología Aplicada del Segura (CEBAS) por su atención y buena disposición cada día.

A mi directora, la Dra. Micaela Carvajal, porque confió en mí desde el primer momento, porque a veces creo que cuando yo aún no sabía que quería realizar una tesis doctoral, ella ya sabía que algún día sería doctora, y sin duda esto ha sido posible gracias a sus enseñanzas en lo laboral y en lo personal, su optimismo, su pasión por este trabajo que hace que no lo sea, su ayuda incondicional día a día y, sobre todo, el cariño que personalmente me ha transmitido desde que empecé a hacer las prácticas hace ahora 7 años.

A la Dra. Mª Carmen Martínez Ballesta por haber ejercido de codirectora. Su ayuda y sus enseñanzas fueron esenciales para mí, sobre todo cuando aún estaba dando mis primeros pasos en el laboratorio y en la ciencia. Todo lo que me enseñó fue clave para haber podido llegar hasta aquí.

En este punto también quiero agradecer al Dr. Jesualdo Fernández Breis, profesor del Departamento de Informática y Sistemas de la Universidad de Murcia, por ser mi tutor académico, estar siempre pendiente de cualquier cosa que he necesitado, y, sobre todo, por permitirme iniciarme como docente.

Sin duda, estos 5 años no hubieran sido lo mismo si no hubiera coincidido con grandes personas por el camino. Muchas gracias a todos los integrantes (pasados y presentes) del Departamento de Nutrición Vegetal y del Grupo Aquaporinas por la amabilidad mostrada en el día a día y toda la ayuda prestada estos 5 años.

No puedo dejar de hacer especial mención a aquellos a los que con mayúsculas podré decir que fueron mis compañeros y AMIGOS de tesis. María, Paula, Álvaro y Juan, gracias por todo, poco tengo que deciros porque creo que después de todo lo vivido sobran las palabras y porque sé que, aunque acaba una etapa para nosotros, nuestros caminos nunca se separarán del todo y siempre nos volveremos a encontrar. Podría extenderme mucho más, pero son miles las anécdotas y las risas que nos llevamos (también lágrimas, momentos duros y despedidas); al escribiros estas palabras han pasado por mi cabeza miles de las cosas vividas, y ahora quiero que cuando leáis esto, hagáis lo mismo, y recordéis todos esos momentos vividos juntos y que sonriáis por dentro como estoy haciendo yo ahora. Gracias por tanto chicos!!. Agradecer también enormemente a Juanjo, sin duda mi doctor de referencia en el laboratorio, en lo bueno y en lo malo siempre estuvo a mi lado. Siempre nos quedaran las risas de nuestro primer café a las 8 de la mañana. También me gustaría extender este agradecimiento al Dr. Diego Moreno, porque desde el principio ha sido un referente científico y un gran apoyo a nivel personal.

A todos aquellos con los que he compartido algún momento durante esta etapa, ha sido mucho tiempo y mucha gente; Paula Marco, Ana, Miriam, María G., Jesús, Yanira, Almu, Joan, Irene, Marisa, Miriam, Fuensanta, Gloria, Fran, Alberto, Pablo, Rafa, Nidia… Este es un trabajo en el que nos acostumbramos a que los compañeros vienen y van, unos están, otros estuvieron, algunos solo unos meses, pero lo importante es que todos aportan algo y dejan huella.

A todos los miembros del SACE y del CAID (César, Alejandro, Toñi, Juana, Silvia, Mª Jesús, Maruja, Tere…) porque está claro que sin ellos esta tesis no hubiera sido lo mismo y su ayuda y conocimiento han sido muy importantes.

También quiero agradecer al Dr. Urban Johanson por acogerme en su grupo como si fuera una más, sin duda hizo que mi estancia en la Universidad de Lund (Suecia) fuera una experiencia muy enriquecedora. Pero sobre todo quiero agradecer a Rebeca porque fue mi mano derecha durante 3 meses, las dos estábamos lejos de casa, pero juntas se notaba un poco menos.

Me gustaría hacer también una rápida mención a todos los maestros y profesores que he tenido durante toda mi vida académica porque he aprendido de todos, muchos fueron los que me dijeron que podría conseguir lo que me propusiera y quiero dejar reflejado mi agradecimiento a todos ellos, a mi colegio y a mi instituto porque sin duda he llegado hasta aquí y soy lo que soy por todo lo vivido allí.

Tengo la suerte de estar rodeada de mucha gente que me quiere y quiero, pero sobre todo tengo la suerte de tener a las mejores amigas del mundo, Ahinoa, Andrea, Rocio y Sara, no podía olvidarme de vosotras, porque me habéis acompañado siempre (de hecho, no recuerdo mi vida sin vosotras, es lo que tiene no recordar ni cuando nos conocimos, allá por 1997), y estos años de tesis, aunque quizás no os hayáis dado cuenta habéis sido un apoyo muy importante para mí. Elena y Alejandro, que hubiera sido de mí sin esos planes de última hora, que fácil ha sido con vosotros desconectar después de un largo "día de tesis", esas cervezas improvisadas a cualquier hora me han dado la vida; habéis sufrido cada momento conmigo, y daba igual que fuera un sábado que un lunes, sois los mejores!. Agradecer también a Inma, David y Adán, es muy fácil hacer equipo con vosotros, todos los momentos y las risas forman también parte de esta aventura. A Marina porque nuestra amistad ya va a ser de esas que duran para toda la vida. Y a todos aquellos que de alguna u otra manera habéis contribuido a que la tesis sea más llevadera, gracias.

Para ir terminando quiero agradecer a TODA MI FAMILIA, a la de sangre y a la que no lo es, pero como si lo fuera. A mis tíos, mis tatas y mis primos, porque sin duda son un pilar fundamental en mi vida. Tampoco quiero olvidarme de mis abuelos, porque, aunque hace mucho tiempo que ya no están, sé que algo de ellos se quedó conmigo. A mi hermana Carmen, porque no hay un momento de mi vida en el que me recuerde sin ella (¡qué suerte el tenerte como hermana!). No puedo dejar de nombrar a mis animalicos, Laika, Casimiro y Linda, por estar y haber estado a mi lado y por darme siempre las mayores de las alegrías. A mis padres, Pepe y Chelo, para mí los mejores del mundo, porque me educaron con los mejores valores y el máximo amor, y solo espero que de "mayor" pueda ser la mitad de buena persona que ellos. Papá, me viste empezar este camino hace 5 años y aunque ahora parezca que no estás para ver como lo termino, yo sé que estás a mi lado y que esto es más tuyo que de nadie.

Para el final he dejado a la persona con la que más tiempo he compartido y comparto, él que ha vivido el proceso como si fuera suyo (podría decirse que juntos hemos hecho dos tesis, que se dice pronto), y el que ha estado conmigo estos últimos 5 años, pero también los 5 anteriores, y espero que todos los que vengan después. Ángel gracias por acompañarme en este camino que es la vida, sin duda sin ti no sería igual de bonita y divertida.

A todos, GRACIAS.

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Memoria presentada para optar al grado de Doctor por la Universidad de Murcia

"Lipid-protein nanovesicles of membrane from *Brassica oleracea* L. var. *italica* as carriers of bioactive compounds".

"Nanovesículas lípido-proteicas de membrana de *Brassica oleracea* L. var. *italica* como transportadores de compuestos bioactivos".

Lucía Yepes Molina

Directoras:

Dra. Micaela Carvajal Alcaraz

Dra. María del Carmen Martínez Ballesta

Tutor:

Dr. Jesualdo Tomás Fernández Breis

Murcia, 2022

INDEX

ABBREVIATIONS

Ab:	Antibody
ANOVA:	Analysis of variance
AQP:	Aquaporins
BBB:	Blood-brain barrier
BDT:	1,2-benzenedithiol
BM:	Broccoli membrane
BSA:	Bovine serum albumin
CCM:	Complete culture medium
CLSM:	Confocal laser scanning microscope
Da:	Dalton
DDS:	Drug delivery system
DLS:	Dynamic light scattering
DRMs:	Detergent resistant membranes
EE:	Entrapment efficiency
ELISA:	Enzyme-linked immunosorbent assay
FDA:	Fluorescein diacetate
FNA:	Sodium fluorescein
FTIR:	Fourier-transform infrared spectroscopy
GRA:	Glucoraphanin
GS:	Gas chromatography
GSL:	Glucosinolates
I3C:	Indole-3-carbinol
IL-6:	Interleukin 6
IL-1β:	Interleukin 1-β
IR:	Infrared
ITCs:	Isothiocyanates
kDa:	Kilodalton
LC-MS:	Liquid chromatography–mass spectrometry
LPS:	Lipopolysaccharide

LUV:	Large unilamellar vesicles
Met:	Methionine
MF:	Microsomal fraction
MLV:	Multilamellar vesicles
miRNA:	Micro RNA
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl:	Sodium chloride
Nf-κB:	Nuclear factor kappa B
NHEK:	Normal human keratinocytes
NO:	Nitric oxide
NPA:	Asparagine-proline-alanine
NPs:	Nanoparticles
NR:	Nile red
Nrf2:	Nuclear factor erythroid 2-related factor 2
PAR:	Photosynthetically active radiation
PDB:	Protein data bank
PdI:	Polydispersity index
PIP:	Plasma membrane intrinsic protein
Pf:	Osmotic water permeability
PM:	Plasma membrane
PMA:	Phorbol 12-myristate 13-acetate
PVP:	Polyvinylpyrrolidone
SC:	Stratum corneum
SEC:	Size exclusion chromatography
SFN:	Sulforaphane
SFN-CYS:	Sulforaphane-cysteine
SUV:	Small unilamellar vesicles
TEM:	Transmission electron microscopy
TNF-α:	Tumour necrosis factor – α
UHPLC-QQQ:	Ultra-high performance liquid chromatography-triple quadruple
UV:	Ultraviolet

RESUMEN

Resumen

España, y concretamente la Región de Murcia tienen una potente industria agrícola, siendo grandes productoras y exportadoras de diversas frutas y verduras. Las especies de la familia *Brassicaceae* tienen una gran importancia agrícola, económica y nutricional. Centrándonos en el brócoli (*Brassica oleracea* L. var. *italica*), en los últimos años se ha incrementado mucho su demanda, ya que posee muchas propiedades beneficiosas para la salud. Tiene un alto contenido en fibra, vitaminas y minerales. Además, es una fuente rica en glucosinolatos (GSL) y compuestos fenólicos, los cuales se han descrito por su papel preventivo en muchas enfermedades, como el cáncer, la diabetes y diversas patologías con un componente inflamatorio.

En concreto, España es la cuarta productora de brócoli a nivel mundial y la Región de Murcia exporta el 70 % de todo el brócoli exportado por España. Las plantaciones de brócoli generan una gran cantidad de subproductos, ya que solo el 25 % de la biomasa total corresponde a partes comestibles y comercializables. El resto de biomasa, hojas y raíces a priori sin valor comercial, es una fuente excelente de multitud de compuestos bioactivos como por ejemplo proteínas, lípidos, vitaminas, compuestos antioxidantes, GSL o isotiocianatos (ITCs). Uno de los compuestos bioactivos del brócoli más estudiados es el sulforafano (SFN), el cual ha sido ampliamente investigado por su potente actividad antioxidante y anticancerígena. Aunque su uso clínico real es limitado debido a su baja estabilidad y biodisponibilidad. En este sentido, el uso de nanotransportadores podría solucionar los problemas relacionados con la estabilidad. Recientemente, los nanotransportadores de origen natural, como pueden ser las vesículas de membrana derivadas de plantas, están recibiendo una gran atención por su potencial para ser empleadas para transportar y liberar de forma controlada y dirigida compuestos bioactivos. Esto tiene un gran interés principalmente en la industria farmacéutica. Este aspecto ha sido de especial relevancia ya que la biocompatibilidad y la aplicación de vesículas vegetales en la industria farmacéutica apenas se había investigado hasta ahora.

Por otro lado, la gran cantidad de residuos y subproductos derivados de la agricultura, suponen un problema severo en Europa en el siglo XXI. En concreto, se ha estimado que 30 % de los productos destinados a la alimentación se convierten en residuos. Esto, se une a diversos factores que agravan la situación como el incremento de la demanda de materias primas, la escasez global de recursos y el impacto en el cambio climático que tiene el manejo de todos los desperdicios. De esta forma, para hacer frente a esta situación, en los últimos años se está promoviendo un nuevo modelo económico más sostenible basado en la economía circular. La Comisión Europea establece que "*una economía circular tiene como objetivo mantener el valor de los productos, materiales y recursos durante el mayor tiempo posible devolviéndolos al ciclo de producción al final de su uso, al tiempo que se busca minimizar la generación de desechos*". Así, el aprovechamiento de los subproductos derivados de la agricultura busca implementar este modelo económico. Dado que la mayoría de los subproductos tienen características nutricionales, funcionales, y nutraceúticas muy interesantes, se ha explorado las posibilidades de su uso para las industrias biotecnológicas.

Teniendo en consideración estas premisas, esta Tesis Doctoral tiene como objetivo principal el estudio de las vesículas de membrana obtenidas de subproductos del brócoli para desarrollar e implementar su uso como nanotransportadores de compuestos bioactivos en aplicaciones de la industria cosmética o farmacéutica relacionada con la piel. Para alcanzar este objetivo global, se han desarrollado tres objetivos específicos, que son los siguientes:

I. Determinar el potencial de las vesículas de membrana extraídas del brócoli para penetrar en las capas internas de la piel y evaluar su capacidad para liberar y suministrar a células de piel los compuestos encapsulados en ellas. Así como determinar la estabilidad de las vesículas en una fórmula cosmética real (***Capítulo I***).
II. Investigar la capacidad de las vesículas de membrana de brócoli para encapsular el compuesto bioactivo SFN y evaluar su actividad en una línea celular de cáncer de piel (***Capítulo II***).

III. Determinar el potencial antiinflamatorio de las vesículas de membrana de brócoli y de encapsulados con SFN en un modelo celular *in vitro* de macrófagos humanos tanto en condiciones normales como en condiciones inflamatorias (***Capítulo III***).

Dado que, durante los últimos años, las vesículas de membrana de origen natural han centrado el foco de muchas investigaciones debido a su naturaleza proteo-lipídica, su tamaño, biocompatibilidad y biodegradabilidad; en esta tesis se exploró la posibilidad de utilizar vesículas de plantas, un aspecto no muy estudiado hasta ahora. Una vez que se tuvieron diferentes tipos de vesículas de membrana de brócoli aisladas, se pasó a evaluar el potencial de las mismas para encapsular compuestos y emplearlas en aplicaciones cosméticas y terapéuticas. De forma que, en el *primer capítulo* de esta tesis investigamos el uso de vesículas de membrana plasmática de raíces aisladas de plantas de brócoli como nanotransportadores. Para ello se determinó la eficiencia de encapsulación y la integridad de las vesículas. Además, se estudió la liberación de la carga de las vesículas en queratinocitos y la penetrabilidad a través de la piel. Los resultados muestran que las vesículas de brócoli presentaron alta estabilidad en relación a su contenido proteico, y alta eficiencia de encapsulación. Por otro lado, se demostró la interacción entre las vesículas y los queratinocitos mediante la liberación de un compuesto fluorescente en las células y mediante la detección de proteínas vegetales en la membrana plasmática de los queratinocitos. Esto demostró las interacciones entre las membranas celulares de especies de reinos biológicos distintos (vegetal y animal). Además, las vesículas de brócoli penetraron y liberaron el compuesto encapsulado en las capas internas de la piel. Así, este sistema obtenido a partir de membranas vegetales se propone como adecuado para su uso en administración transdérmica de fármacos o compuestos bioactivos.

Posteriormente, se plantearon estudios para investigar el uso de vesículas obtenidas de hojas de brócoli para encapsular un compuesto bioactivo como es el SFN, el cual tiene propiedades antioxidantes y anticancerígenas. En primer lugar y abordado en el *segundo capítulo*, se estudió el poder anticancerígeno de

encapsulados de SFN en las vesículas de brócoli. Se llevó a cabo un análisis fisicoquímico para caracterizar las vesículas a través de diferentes enfoques: dispersión dinámica de luz, microscopía electrónica de transmisión, análisis de la permeabilidad osmótica y análisis proteómico. Para testar el poder anticancerígeno, se aplicaron diferentes concentraciones de vesículas con y sin el SFN en una línea celular de melanoma durante 24 h para estudiar la citotoxicidad y la expresión génica. Cuando se aplicó el SFN encapsulado en las vesículas, se observó una disminución en la proliferación de las células y se produjo una reducción de la expresión génica de marcadores asociados a cáncer y un aumento de AQP3. Por otro lado, se estudió el metabolismo del SFN dentro de las células y se determinó una mayor cantidad se SFN en el interior de las células cuando se aplicaba de forma encapsulada. Los resultados mostraron que el SFN encapsulado fue mejor absorbido por las células de melanoma proporcionando productos de metabolismo.

Para continuar profundizando en las posibles aplicaciones que pueden tener las vesículas de membrana obtenidas del brócoli, en el *tercer capítulo* se investigó sobre un aspecto farmacéutico en relación con la inflamación. Esta es una patología clave en el desarrollo de varias enfermedades con una mortalidad considerable y, por lo tanto, con alto interés en su investigación. Para ello, se determinaron los mecanismos de actuación de los encapsulados de SFN en una línea celular de macrófagos, dado el interés creciente para encontrar nuevos fármacos antiinflamatorios no tóxicos. Primeramente, se realizó un modelado *in vitro* de la liberación del SFN encapsulado en las vesículas y los resultados obtenidos mostraron que se producía una liberación en dos fases, una más rápida y otra más lenta, lo que se relacionó con que parte del SFN podía interaccionar con las proteínas transmembrana, como las acuaporinas (AQPs). Determinándose mediante un estudio de "*blind docking"* la posible interacción específica del SFN con una acuaporina de plantas a través de los motivos NPA conservados de la misma. En cuanto a los ensayos celulares realizados en un modelo celular de macrófagos humanos en condiciones normales e inflamatorias, se demostró la acción antiinflamatoria de las vesículas cargadas de SFN, ya que se observó una

disminución de las interleucinas cruciales para el desarrollo de la inflamación (TNF-α, IL-1β e IL-6). Además, estos resultados también mostraron que las vesículas por sí mismas tenían propiedades antiinflamatorias, abriendo la posibilidad de nuevas líneas de investigación para estudiarlas no solo como transportadores, sino también como compuestos bioactivos y fármacos en sí mismos.

Así, los resultados obtenidos en esta Tesis Doctoral concluyeron lo siguiente:

I. Las vesículas de membrana plasmática de raíces de brócoli mostraron una alta eficiencia de encapsulación empleando dos tintes como compuestos modelo y presentaron una buena estabilidad en condiciones *in vitro* y en una fórmula cosmética real durante un año, manteniendo una cantidad de proteína suficiente y una funcionalidad adecuada.
II. Las vesículas de membrana plasmática de raíces de brócoli liberaron un compuesto fluorescente encapsulado en queratinocitos humanos *in vitro* en cultivo celular y en las capas internas de un disco de piel de cerdo, siendo por tanto capaces de cruzar la barrera impermeable superficial de la piel (estrato córneo).
III. La aplicación en cultivos celulares (una línea celular de queratinocitos humanos) de vesículas de membrana plasmática derivadas de raíces de brócoli permitieron mostrar interacciones entre las membranas celulares vegetales y humanas, destacando el papel de las AQPs de las vesículas de brócoli en esta interacción.
IV. Las vesículas de fracción microsomal de hojas de brócoli son eficientes para encapsular el compuesto bioactivo sulforafano (SFN), el isotiocianato más importante del brócoli. Tras la encapsulación, las propiedades de las vesículas no cambiaron, manteniendo el tamaño, la polidispersidad, el potencial Z y la permeabilidad osmótica al agua. Además, el SFN no alteró su bioactividad en ensayos *in vitro* tras la encapsulación.
V. La caracterización de la composición de las vesículas obtenidas de fracción microsomal de hojas de brócoli reveló que contienen compuestos bioactivos

como ITCs e índoles (sulforafano (SFN), erucina, iberina o indol-3-carbinol (I3C)), y proteínas asociadas con actividad antioxidante, lo que es importante en la bioactividad propia de las vesículas.

VI. El SFN encapsulado en vesículas de membrana derivadas de hojas de brócoli mostró una elevada actividad de inhibición del desarrollo de melanocitos (línea celular SK-MEL-28) cuando se aplicó durante 24 h en cultivo celular y desencadenó un aumento del gen p53, que da lugar a una proteína supresora de tumores.

VII. El SFN, tanto en forma libre como encapsulada, y las vesículas de membrana de hojas de brócoli aumentaron la expresión del gen AQP3 (acuaporina 3, que se encuentra en la capa de células basales de la epidermis) cuando se aplicó durante 24 horas en un cultivo de células de melanoma. La AQP3 fue señalada como un marcador importante en el cáncer.

VIII. Al comparar la aplicación de SFN no encapsulado y encapsulado en vesículas de membrana de hojas de brócoli en un cultivo de células de melanoma se vio una mejor entrega de SFN en las células cuando el compuesto se aplicó en la forma encapsulada.

IX. Las vesículas de la fracción microsomal de las hojas de brócoli se estandarizaron mediante filtración y se obtuvo un tamaño homogéneo de unos 200 nm, que no cambió cuando se encapsuló el SFN.

X. Mediante un ensayo de liberación *in vitro* se determinó que la liberación de SFN encapsulado en vesículas de membrana de hojas de brócoli ocurre en dos fases. En primer lugar, el SFN se liberó más rápidamente, posiblemente a través de la membrana lipídica, y después de forma más lenta y controlada, lo que podría ser causado por la interacción con las proteínas de la membrana.

XI. Mediante un estudio *in silico* se determinó una interacción específica entre el SFN y las acuaporinas PIP de plantas presentes en las vesículas de membrana a través de los motivos conservados NPA de las AQPs.

XII. Las vesículas de membrana de hojas de brócoli con SFN encapsulado mostraron un importante potencial antiinflamatorio en un modelo *in vitro* de macrófagos humanos debido a la inhibición de los niveles de citocinas inflamatorias TNF-α, IL-6 e IL-1β con citotoxicidad muy baja.

SUMMARY

Summary

Spain and specifically the Region of Murcia have a powerful agricultural industry, being large producers and exporters of various fruits and vegetables. Species from the *Brassicaceae* family have great agricultural, economic, and nutritional importance. Focusing on broccoli (*Brassica oleracea* L. var. *italica*), its demand has increased a lot in recent years, as it has many health beneficial properties. Broccoli has high fibre content, vitamins, and minerals. Besides, it is also a rich source of glucosinolates (GSL) and phenolic compounds, which have been described for their preventive role in several diseases such as cancer, diabetes, and other pathologies with an inflammatory component.

Specifically, Spain is the fourth largest producer of broccoli in the world, and the Region of Murcia exports 70 % of all the broccoli exported by Spain. Broccoli crops produce a large amount of by-products, as only 25 % of the total biomass corresponds to the edible and marketable parts. The remaining biomass, leaves and roots, a priori of no commercial value, are an excellent source of a multitude of bioactive compounds such as proteins, lipids, vitamins, antioxidant compounds, GSL, or isothiocyanates (ITCs). One of the most studied bioactive compounds in broccoli is sulforaphane (SFN), which has been extensively studied for its potent antioxidant and anticarcinogenic activity. However, its actual clinical use is limited due to their low stability and bioavailability. In this sense, the use of nanocarriers could solve stability-related problems. Recently, nanocarriers from natural sources such as plant-derived membrane vesicles are receiving a great deal of attention for their potential to be used to transport and release bioactive compounds in a controlled and targeted manner. This is of great interest mainly in the pharmaceutical industry. This aspect has been of special relevance since the biocompatibility and application of plant-derived vesicles in the pharmaceutical industry had hardly been investigated until now.

On the other hand, the large amount of waste and by-products from agriculture is a severe problem in Europe in the 21st century. Specifically, it has been estimated that about 30 % of food products become waste. This is combined with a number of factors that aggravate the situation, such as the increased demand for raw materials, the global scarcity of resources, and the climate impact of waste management. In order to address this situation, a new and more sustainable economic model based on the circular economy has been promoted in recent years. European Commission states that "*a circular economy aims to maintain the value of products, materials, and resources for as long as possible by returning them into the product cycle at the end of their use, while minimising the generation of waste*". Thus, the exploitation of by-products derived from agriculture seeks to implement this economic model. Since most by-products have very interesting nutritional, functional, and nutraceutical characteristics, the possibilities of their use in biotechnological industries have been explored.

Taking these premises into consideration, the main objective of this Doctoral Thesis is the study of membrane vesicles obtained from broccoli by-products in order to develop and implement their use as nanocarriers of bioactive compounds in applications for the cosmetic or pharmaceutical industry related to the skin. To achieve this overall objective, three specific objectives have been developed, which are as follows:

I. To elucidate the potential of membrane vesicles extracted from broccoli to penetrate the inner layers of the skin and evaluate their ability to release and deliver encapsulated compounds to skin cells. As well as to determine the stability of the vesicles in a real cosmetic formulation (***Chapter I***).

II. To elucidate the capacity of membrane vesicles from broccoli to encapsulate the bioactive compound SFN and evaluate their activity in a skin cancer cell line (***Chapter II***).

III. To determine the anti-inflammatory potential of broccoli membrane vesicles and encapsulation with SFN in a human-macrophage-like *in vitro* cell model under both normal and inflammatory conditions (***Chapter III***).

Since in recent years, membrane vesicles of natural origin have been the focus of many investigations due to their proteo-lipid nature, size, biocompatibility, and biodegradability; this thesis explored the possibility of using plant vesicles in an applied form, an aspect that has not widely been studied. Once different types of broccoli membrane vesicles were isolated and characterised, the potential of these vesicles to encapsulate compounds for cosmetic and therapeutic applications was evaluated.

In the *first chapter* of this thesis, we investigated the use of isolated plasma membrane vesicles from roots of broccoli plants as nanocarriers. For that, entrapment efficiency and integrity of vesicles were determined. In addition, the release of vesicle cargo in keratinocytes and penetrability through the skin were studied. The results showed that broccoli vesicles presented high stability in relation to their protein content, and high entrapment efficiency. Furthermore, the interaction between vesicles and keratinocytes was demonstrated by releasing a fluorescent compound into the cells and by detecting plant proteins in the plasma membrane of keratinocytes. These proved interactions between cell membranes of species from different biological kingdoms (plant and animal). Besides, broccoli vesicles penetrated and released the encapsulated compound into the inner layers of the skin. Thus, this system obtained from plant membranes is proposed as suitable for using in the transdermal delivery of drugs or bioactive compounds.

Subsequently, studies were proposed to investigate the use of vesicles obtained from broccoli leaves to encapsulate a bioactive compound such as SFN, which has antioxidant and anticarcinogenic properties. Firstly, in the *second chapter* the anti-cancer activity of SFN encapsulated in broccoli vesicles was studied. A physicochemical analysis was carried out to characterise membrane vesicles through different approaches: dynamic light scattering, transmission electron microscopy, water permeability analysis, and proteomic study. To test the anti-

cancer activity, different concentrations of vesicles with and without SFN were applied to a melanoma cell line for 24 h to study cytotoxicity and gene expression. When encapsulated SFN was applied, a decrease in cell proliferation was observed, and there was a reduction in gene expression of cancer-associated markers and an increase in AQP3. On the other hand, the metabolism of SFN inside the cells was studied and a higher amount of SFN inside the cells was determined when it was applied in an encapsulated form. The results showed that encapsulated SFN was better absorbed by melanoma cells resulting in products of SFN metabolism and a reduction of molecular cancer markers.

To further explore the possible applications of membrane vesicles obtained from broccoli, the *third chapter* it was investigated a pharmaceutical aspect related to inflammation. Inflammation is a key pathology in developing several diseases with considerable mortality, and therefore it has a high research interest. To this end, the action mechanisms of SFN encapsulates in a macrophage cell line were determined, given the growing interest in finding new non-toxic anti-inflammatory drugs to treat inflammation. First, *in vitro* modelling of encapsulated SFN release from vesicles was carried out, and obtained results showed that there was a two-phase release, one faster and one slower, this last, related to a part of SFN that could interact with transmembrane proteins, such as AQPs. A blind docking study determined the possible specific interaction of SFN with a plant aquaporin through its conserved NPA motifs. Cellular assays performed in a human macrophage cell model under normal and inflammatory conditions demonstrated the anti-inflammatory action of SFN-loaded vesicles, as a decrease in interleukins crucial for the development of inflammation (TNF-α, IL-1β, and IL-6) was observed. Furthermore, these results also showed that vesicles themselves had anti-inflammatory properties, opening up the possibility of new research lines to study them not only as transporters but also as bioactive compounds and drugs.

Hence, the results obtained in this Doctoral Thesis concluded the following:

I. Broccoli root plasma membrane vesicles showed high entrapment efficiency using two dyes as model compounds, and showed good stability

under *in vitro* conditions and in a real cosmetic formulation for one year, maintaining enough amount of protein for adequate functionality.

II. Broccoli root plasma membrane vesicles encapsulating a fluorescent dye, released it to both cell culture human keratinocytes and in the inner layers of a pig skin disc. Thus, the vesicles were able to cross the PM of keratinocyte and the impermeable surface barrier of the skin (stratum corneum).

III. The application of broccoli-derived membrane vesicles in cell cultures (a human keratinocyte cell line) allowed to show interactions between plant and human cell membranes, highlighting the role of AQPs in this contact. Crossover interactions between both kingdoms leading open many lines of investigation and numerous potential applications.

IV. Microsomal fraction vesicles from broccoli leaves are very efficient in encapsulating the bioactive compound sulforaphane (SFN), being the most important isothiocyanate in broccoli. After encapsulation the properties of vesicles did not change, maintaining size, polydispersity, Z-potential, and osmotic water permeability. Furthermore, SFN did not alter its bioactivity *in vitro* assays upon encapsulation.

V. Analysis of the composition of vesicles obtained from broccoli leaves microsomal fraction revealed that they contain bioactive compounds such as ITCs and indoles like sulforaphane (SFN), erucin, iberin or indole-3-carbinol (I3C), and proteins associated with antioxidant activity, important in self-activity of vesicles, and proteins related to binding activity, which could be key in the fusion with target cells.

VI. SFN encapsulated in broccoli membrane vesicles showed high inhibition activity of melanocyte development when applied for 24 h in cell culture and triggered an increase of the p53 antioncogene, which gives rise to a tumour suppressor protein.

VII. SFN in both free and encapsulated form, and broccoli membrane vesicles, up-regulated AQP3 (aquaporin 3 found in the basal cell layer of the epidermis) gene expression when they were applied for 24 h in a melanoma cell culture. AQP3 was pointed to as important marker in cancer.

VIII. Comparison of application of SFN non encapsulated and encapsulated SFN in broccoli membrane vesicles to melanoma cell culture (cell line SK-MEL-28) revealed better delivery of SFN into the cells when the compound was applied in the encapsulated form.

IX. Microsomal fraction vesicles from broccoli leaves were standardized through filtration and a homogeneous size about 200 nm was obtained, which did not change when SFN was encapsulated.

X. An *in vitro* release assay determined that the release of SFN encapsulated in broccoli membrane vesicles occurred in two phases. First, SFN was released more rapidly, possibly through lipid membrane, and then in a slower and more controlled manner, which could be due to interaction with membrane proteins.

XI. An *in silico* study determined a specific interaction between SFN and plant PIP aquaporins present in membrane vesicles via the conserved NPA motifs of aquaporins, which may enhance stabilization of the compound in the vesicles.

XII. The anti-inflammatory potential of SFN-loaded broccoli membrane vesicles in a human macrophage-like cell model *in vitro* (differentiated HL-60 cells) due to a significant inhibition of the inflammatory cytokines TNF-α, IL-6, and IL-1β levels with very low cytotoxicity.

INTRODUCTION

Introduction

1. Revaluation of agricultural by-products

The waste of products destined for the food industry is a severe problem in Europe in the 21st century. Around 30 % of products for human consumption become waste, and in the case of fruits and vegetables, the waste rises to 46 % (Caldeira et al., 2019). In addition to the products directly intended for food, a large amount of waste without any value in the food market is generated in agriculture, such as leaves, stems, or roots from crops, which further increase the waste generated by the agricultural industry. In recent years to aim this situation, a more sustainable economic model known as the circular economy has been promoted. According to the European Commission, "*a circular economy aims to maintain the value of products, materials, and resources for as long as possible by returning them into the product cycle at the end of their use, while minimising the generation of waste*". The reasons for developing such an economy model are the increased demand for raw materials, the global scarcity of resources, and the climate impact of waste management.

The term bioeconomy has been established for economic processes with a biologic base. This economic system consists of integrating agriculture, health and industry models; under the premise of reducing the carbon footprint, using alternative fuels, and giving added value to the waste. In this context, the management of waste generated during agricultural production entails an environmental problem. Although, taking advantage of these wastes is possible due to the nutritional, functional, and nutraceutical characteristics of most agricultural waste (Fernández-Ochoa et al., 2021).

From agricultural by-products can be purified biomolecules such as proteins, lipids, vitamins, fibres, antioxidants, glucosinolates (GSL), and others with significant potential and numerous biotechnological applications. Thus, the aim is to reduce the amount of waste and implement the concept of bioeconomy.

1.1. Importance of broccoli crops by-products

Plants belonging to the family *Brassicaceae* were the first domesticated plants, and their origin dates back to 2500 BC. Specifically, the origin of *Brassica oleracea* L. var. *italica* (broccoli) (Figure 1A) is located in the Middle East (Minor Asia, Lebanon, and Syria), and its cultivation spread throughout Europe (Spain, France, Greek, Denmark, and England) from the XVI century. Although, it was not until the last century that broccoli became popular in various countries. Specifically, in Spain large scale broccoli cultivation began in the early 1970s. Currently, Spain is the fourth-largest broccoli producer worldwide after China, India, and EEUU. In our country, especially in the Region of Murcia, broccoli has become the horticultural crop that has increased the most, being Murcia the main broccoli exporter in Spain. Region of Murcia exported 69.85% of all broccoli exported by Spain in 2018 («Proexport», 2018), and the latest published data showed a total production of 251,268 tons, which represents 45.56% of all the broccoli produced in Spain («Ministerio de Agricultura, Pesca y Alimentación», 2021) (Figure 1B). Thus, broccoli crops are an important piece in the agricultural economy of our region.

Species from the *Brassicaceae* family have importance from both agronomic and economic points of view. In addition to producing vegetables, *Brassica* species are used to obtain oils, condiments, and animal food. Besides, different studies have shown that brassicas could be used for phytoremediation due to their high tolerance to heavy metals (Rizwan et al., 2018), and also, some species of the *Brassica* genus have a possible application in the manufacture of biofuels (Fatima et al., 2021).

From a nutritional point of view, *Brassica* species have several highlights. Specifically, the demand for broccoli cultivation is due to its beneficial properties and its high nutritional value. Broccoli is basically made up of water and fibre, and is suitable in weight loss diets due to its low caloric content. Besides, broccoli is a source of a large number of vitamins (A, C, E, B1, and K) and minerals (Ca, Mg, Na, K, F, Zn, and Se), which are essential in the formation of collagen, bones, or white and red blood cells (Jeffery et al., 2003; Moreno et al., 2006). Also, many

bioactive compounds are present in broccoli, which are metabolites and chemical compounds from the secondary metabolism of plants as GSL and phenolic compounds. These compounds have properties such as the antioxidant capacity to prevent some diseases such as different types of cancer, diabetes, or inflammatory illness, in addition, to increasing infection resistance or regulating the correct development of the nervous system (Jeffery and Araya, 2009).

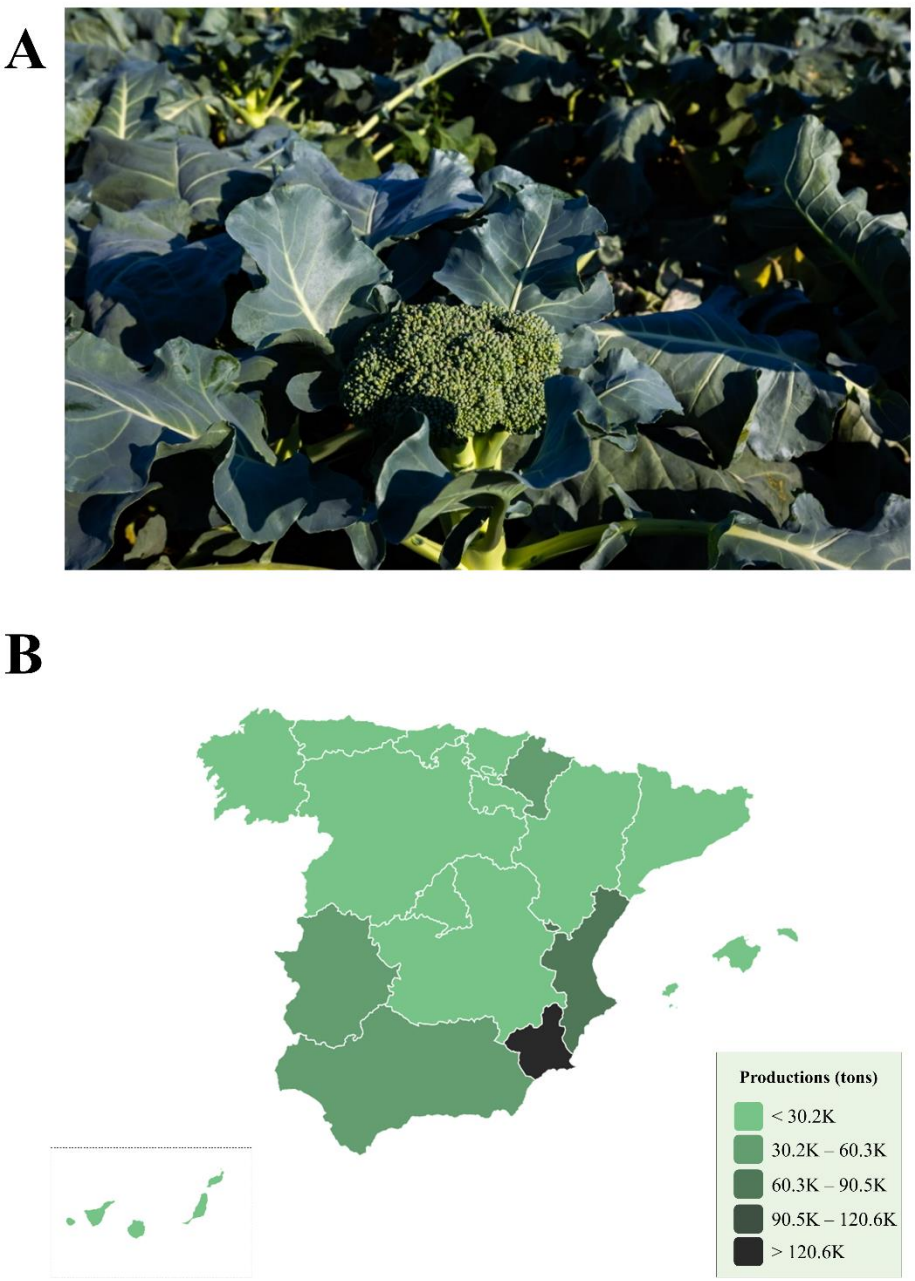

Figure 1. *Brassica oleracea L. var. italica (broccoli) crops (A). Map of Spain of production of broccoli (tons) by regions (B).*

1.2. Biotechnological applications of broccoli crops by-products

Broccoli crops represent a great agronomical by-products source because marketable production is only 25% of total biomass (Fink et al., 1999). The remaining 75% are by-products, mainly leaves, stems and roots, a priori without economic and commercial value. However, following the established path based on the circular economy, different uses have been found to take advantage of agricultural residues from broccoli, such as obtaining bioactive ingredients or vesicles from plant membranes for use as nanocarriers (Domínguez-Perles et al., 2010; Martínez Ballesta et al., 2016). These components could benefit the drug, cosmetic, and food industries.

1.2.1. Bioactive compounds in broccoli

Broccoli is a source of nutraceutical compounds, both broccoli florets (edible part) and by-products have similar chemical composition (Drabińska et al., 2018). Therefore, the use of broccoli by-products is a viable way to obtain these nutraceutical compounds without the need to use the edible and commercial part of broccoli crops. Among all bioactive compounds present in broccoli plants could be highlighted: GSLs and isothiocyanates (ITCs), and phenolic compounds, along with flavonoids, are the most important group due to their abundance in the diet and their health benefits as their antioxidant capacity. (Panche et al., 2016).

Thus, bioactive compounds can be obtained from different broccoli by-products to be used in different fields. In this sense there are two approaches. For medical applications, pure compounds are needed and this implies more complex and expensive purification processes. But applications can be found in which extracts can be used as complex mixtures of different compounds, whose purification is more affordable. So that, in one way or another broccoli crops by-products can be used to the fullest. Consequently, these extracts could be used in applications related to both cosmetic and food industry.

1.2.1.1. Glucosinolates

GSL are sulfurized compounds from secondary metabolism that plant use principally as defence against biotic (herbivory, fungal, bacterial and/or viral infection) and abiotic stresses (salinity, temperature, UV or metals). In fact, plants increase the production of GSL under these unfavourable conditions (Zaghdoud et al., 2012; Martínez-Ballesta et al., 2013; Cocetta et al., 2018).

Regarding chemical structure, GSL are anions composed of a β-thioglucose, a sulfonated oxima and a side chain derived from amino acids. Based on this amino acid, GSL are classified in three groups: aliphatics (derived from Ala, Leu, Ile, Met or Val), aromatics (Phe or Tyr), and indolics (Trp) (Wittstock and Halkier, 2002). The total number of identified GSL is between 88 and 137, since 88 have been successfully characterized by modern and accurate methods (NMR and MS), and 49 structures have been partially identified (Blažević et al., 2020). In broccoli 13 different types of GSL have been found (Wang et al., 2012) and glucoraphanin (GRA) (Figure 2A) is the most abundant GSL, normally constituting 50% of the total GSL (Jeffery et al., 2003).

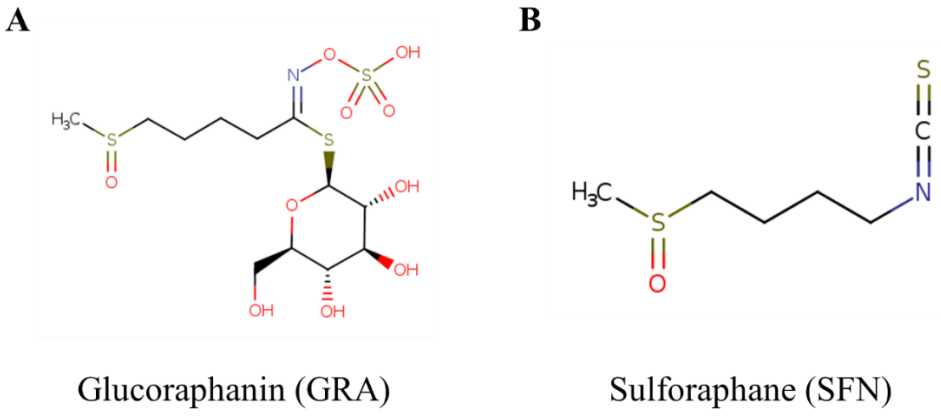

Figure 2. *Structure of the glucosinolate glucoraphanin (A) and the isothiocyanate sulforaphane (B).*

A large number of epidemiological studies have linked *Brassicas* consumption and specifically GSL with a reduced risk of certain illnesses, such as cancer, diabetes, obesity or various pathologies with an inflammatory component. However, it is currently known that most of the biological activity of GSL comes from their breakdown products, ITCs.

1.2.1.2. Isothiocyanates: sulforaphane

ITCs are hydrolysis products from GSL by action of the myrosinase (EC 3.2.3.1). Myrosinase in an enzyme which is stored in plant cells or separate intracellular compartments of GSL, but against a damage this enzyme gets in touch with GSL and myrosinase-catalysed GSL hydrolysis is initiated (Kelly et al., 1998). Moreover, GSL could also been hydrolysed by the gastrointestinal tract microflora, which is essential to obtain the health benefices from GSL ingested in the diet through vegetables belonging to *Brassicaceae* family (Tian et al., 2018). Each GSL gives a different and specific ITC. One of the most important ITC in broccoli is sulforaphane (SFN) (Figure 2B), which is derived from GRA hydrolysis.

Regarding their bioactivity in human, ITC have been widely studied due to their powerful antioxidant and anticarcinogenic activity. Specifically, SFN has been described for its relevant role both in the prevention and treatment of different types of cancer, although most studies carried out with this compound in this sense are preliminary and early stage assays (Quirante-Moya et al., 2020). Protective function of SFN is due to its action in different mechanisms and pathways. SFN is implicated in the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which on the one hand will induce phase II enzymes important in the protection against oxidative stress, and on the other hand, genes encoding enzymes capable of inhibiting certain proinflammatory factors will be induced (Dinkova-Kostova and Kostov, 2012) (Figure 3A). Also, SFN can strongly arrest cell cycle in G2/M and induce apoptosis in different cancer cell lines (Cheng et al., 2016), carry out an epigenetic regulation regarding histones and DNA methylations (Fuentes et al., 2015), and inhibit angiogenesis and metastasis (Liu et al., 2017). In addition, via Nrf2, SFN acts through modulation of nuclear factor kappa B (Nf-κB), which is a transcription factor that regulates genes involved in immune and inflammatory responses. SFN inhibits translocation of Nf-κB into the nucleus and so SFN can ameliorate inflammation by inhibiting the Nf-κB signaling pathway (Ruhee and Suzuki, 2020) (Figure 3B).

In addition to cancer, the use of ITCs had been proposed to treat or prevent other diseases, mainly all those with an inflammatory component such as diabetes, obesity or endometriosis (Baenas et al., 2016; García-Ibañez et al., 2020). As well as it has been seen that the SFN and other ITC have protective effect against related central nervous system diseases (Dinkova-Kostova and Kostov, 2012).

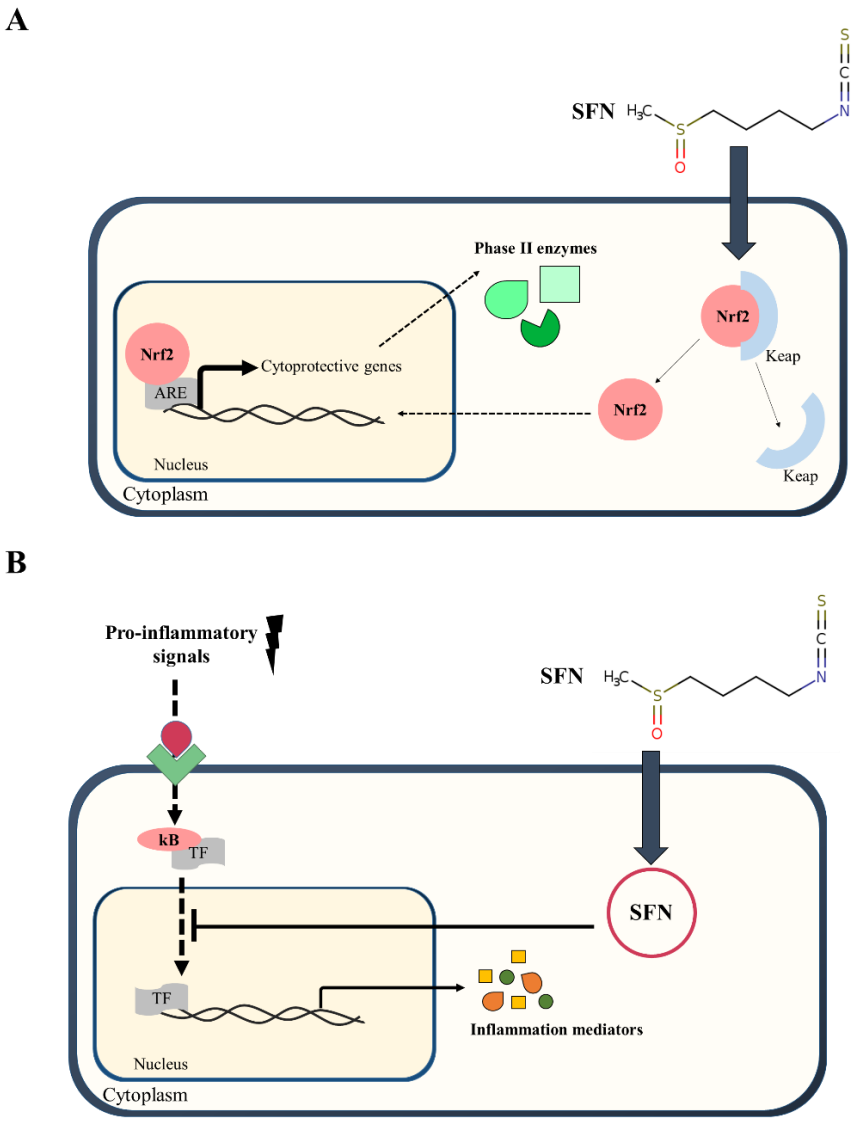

Figure 3. *Scheme of action of sulforaphane (SFN). SFN acts via Nrf2 (nuclear factor erythroid 2-related factor 2) pathway (A), where a direct activation of phase II enzymes transcription is triggered by SFN action. SFN modifies cysteine residues of Keap (Kelch-like ECH-associated protein) and Nrf2 is delivered and translocated into the nucleus, where Nrf2 interacts with antioxidant response elements (ARE) to activate the transcription machinery of genes encoding phase II enzymes. In addition, SFN act via Nf-κB (nuclear factor kappa B) (B), through inhibition of the translocation of transcription factors (TF) into the nucleus, which under basal conditions are sequestrated into the cytosol, but when pro-inflammatory ligands bind to its receptors, TF are able to translocate into the nucleus and transcript several inflammatory mediators.*

1.2.2. Membrane vesicles

Membrane vesicles purified from different natural sources as plant material are receiving considerable attention as nanocarriers for transporting and delivering compounds into target cells. Various studies have shown cross-reactions and similitudes between plants and animal membranes, which patents the possibility of using plant vesicles to transport compounds to animal cells. In this sense, broccoli by-products (root and leaves) are a suitable source to get membrane vesicles for different biotechnological applications. Membrane vesicles are composed mainly of lipids and proteins; many are transporters and channel proteins. This type of proteins (transmembrane) may be involved in the stability and delivery of entrapment molecules (Martínez Ballesta et al., 2016). Among membrane proteins are aquaporins (AQPs), which are relevant in membrane vesicles obtained from plant material. Due to their function as water transporter, AQPs will be fundamental in the membrane permeability adjustment, the maintenance of the osmolarity, and the hydration status of the membranes (Martínez-Ballesta and Carvajal, 2016). *These issues are explained in detail in sections 3. "Plant-derived membrane vesicles" and 4. "Broccoli-derived membrane vesicles: background"*.

2. Nanoencapsulation systems

Nanoencapsulation is a technology that entails the incorporation of substances (active agents) at the nanoscale range (10 to 1000 nm) (Patra et al., 2018) (Figure 4) in vesicular structures to stabilize, protect, transport, and release encapsulated compounds at the right time, in a safe manner, and usually at a specific target site. These vesicular structures or nanocarriers, which can be of different types and nature, have been highlighted for their potential to improve multiple biotechnological applications in several fields: medicine, cosmetics, agriculture, and food (Cano-Sarabia and Maspoch, 2015). The nanoencapsulation field is an extensive area of research, and several aspects must be addressed. When choosing

and studying nanoencapsulation systems, the essential characteristics are size, entrapment efficiency, storage stability, and *in vitro* or *in vivo* release properties.

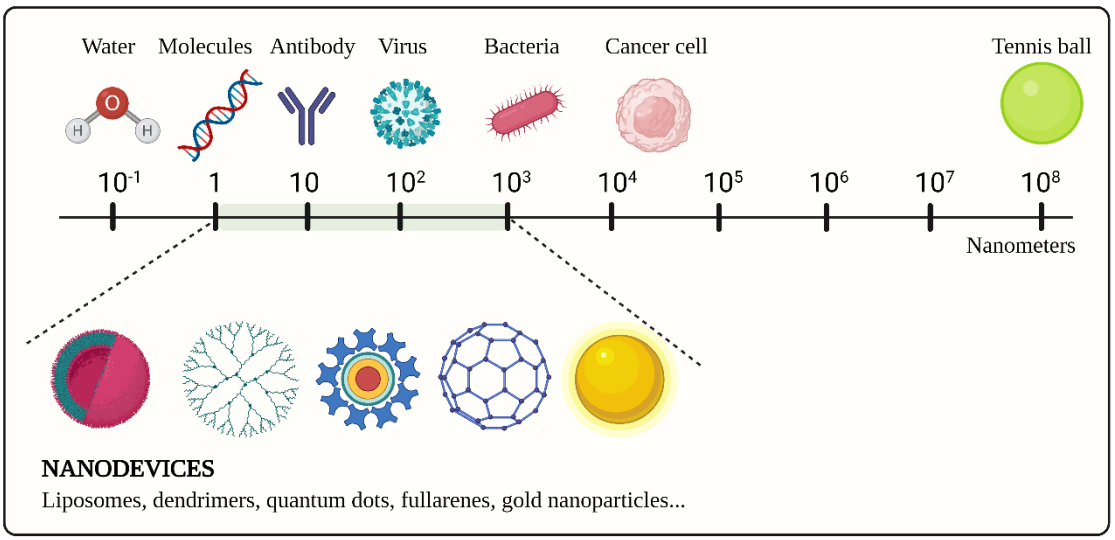

Figure 4. *Nanoscale diagram: from macro-materials to atoms. Nanodevices that are of interest in nanotechnologies are at 1-1000 nm. Figure adapted from* Al-Hakkani, (2020).

2.1. Interest in nanoencapsulation

The development of new and improved nanoencapsulation systems or drug delivery systems (DDS) has aroused great interest in both industry and research. The encapsulation of bioactive compounds with beneficial properties for human health is relevant when drugs or cosmetics are being developed. On the one hand, due to the *in vivo* and *in vitro* instability of many compounds with biotechnological interest and, on the other hand, the difficulty of these compounds to cross biological barriers.

2.1.1. A strategy for improving bioactive compound characteristics

Natural bioactive compounds generally have several health-promoting properties and potent *in vitro* pharmacological activities. Although, the translation of this activity into clinical or *in vivo* therapeutic effects is problematic. These compounds are chemically unstable due to their poor water-solubility and susceptibility to oxidative degradation (Pachuau et al., 2021). In addition, they display rapid systemic clearance *in vivo* conditions, low oral absorption, and poor

bioavailability, the last of the properties is one of the most important that bioactive compounds need to exert any beneficial effects *in vivo*. Bioavailability is a process that includes different phases: liberation, absorption, distribution, metabolism, and elimination. Therefore, without this characteristic, the application of free bioactive compounds is limited in drug formulation, cosmetic, or food industry. On the other hand, environmental factors (temperature, pH, light, oxygen, and enzymes) also contribute to the degradation *in vitro* and *in vivo* of these bioactive compounds (Pachuau et al., 2021). Some very studied natural compounds by their beneficial properties, such as curcumin, quercetin, or SFN, presented the problems mentioned above (Anand et al., 2007; Cai et al., 2013; Zambrano et al., 2019). As regard SFN, it has a short half-life of 2 h *in vivo* conditions (Hu et al., 2004), is degraded in an aqueous medium at 50 – 100 ºC aggravated by a rise in pH (Jin et al., 1999), and is rapid clearance and metabolism which affect its bioavailability (Do et al., 2010).

Therefore, encapsulation is a viable alternative to stabilize compounds *in vitro*, preserve their quality, improve bioavailability, or enhance their application in cosmetic, food, or drug formulations. Thus, previous studies have revealed encapsulation as an effective method to protect molecules like SFN from degradation. Different types of nanocarriers have been explored as delivery systems for SFN to address this objective, such as albumin microspheres, polymers (gelatin, gum arabic, or pectins), and micelles (Yuanfeng et al., 2021). Improvements have been shown when SFN is encapsulated compared to when it is in a free form. Some of these improvements are: 20% increase when stored at high temperatures (Tian et al., 2015), alginate based microspheres enhanced stability of SFN at pH 7.4 (Wang et al., 2011), SFN encapsulated in BSA based microspheres enhanced tumour inhibition *in vivo* compared to free SFN (Do et al., 2010), and magnetic microspheres increased tumour growth inhibition when SFN was encapsulated compared to the free SFN (Enriquez et al., 2013).

2.1.2. A strategy for overcoming biological barriers

Encapsulation of bioactive compounds not only may improve the properties mentioned above but also is an alternative to facilitate bioactive compounds across

biological barriers, which are necessary components that protect the body from invading pathogens or foreign material, and maintaining homeostasis. Bioactive compounds have a priori a positive effect, but they are strange elements for the organism, and these compounds will not cross biological barriers easily. Active agents may cross the biological system, entering the blood circulation and reaching the target site to exert their biologic effects. Thus, nanoencapsulation of bioactive agents is, therefore, an alternative to bypass restrictions offered by biological barriers, including the immune system, biological hydrogels, cell trafficking pathways, and tissue barriers (Finbloom et al., 2020); with a special focus on the latter, which is explained in more detail below.

- The **immune system** will recognise drugs/bioactive compounds as foreign substances after their application and an immune response will be triggered. Phagocytes will internalize drugs and provoke rapid particle clearance and low treatment efficacy. Two alternatives are proposed to prevent this: the design of carriers with immune-evasive properties (Huynh et al., 2010), and the design of carriers to either activate or suppress the immune system for a given application. For example, nanoparticles (NPs) for immunostimulation have been employed against cancer and infections, whereas immunosuppressor NPs have been employed for inflammatory disorders like rheumatoid arthritis or diabetes (Feng et al., 2019).

- **Biological hydrogels** are throughout the body. Drugs to reach the target sites must navigate in hydrogels, which limit the penetration of particles and, therefore, an effective drug delivery. The most studied biological hydrogels are mucus (Lai et al., 2009) and bacteria hydrogels (Gupta et al., 2019), so hydrogels are biologic barriers and drug targets. In the design of drug carriers, a decision must be made between mucoadhesion and mucus penetration. Regarding materials, mucus-penetrating carriers have been developed with silica, polymers, and liposomes. Carriers with characteristics to cross these types of barriers are desirable for oral drug delivery applications (Fox et al., 2015).

- The last barriers that drugs have to cross until exert their functions are the **cellular trafficking and cell uptake** (Finbloom et al., 2020). Several DDS have been design to improve the PM passage and to avoid endolysosomal sequestration. In this sense, enormous efforts are being done for developing nanocarriers with a modified surface to inducing cytosolic uptake of NPs. For example, surface of DDS modified with cell-penetrating peptides can permeabilise cell membranes and improve the transport of encapsulated drug or intact NPs into the cytosol (Farkhani et al., 2014).

Tissues barriers: skin

Tissue barriers are the most widespread biological barriers to drug delivery to the target site. These barriers are characterized by their composition based on closely and packed cells, which limit drug penetration into the blood flow inhibiting on the one hand paracellular transport between cells and on the other hand transcytosis across the cell (González-Mariscal et al., 2005). There are different tissues barriers, but the most important and studied in drug delivery are blood-brain barrier (BBB), enterocytes and skin. BBB is a structure that limits the entry of small molecules into the healthy brain and achieving the local delivery of drugs into the brain is difficult. Therefore, developing DDS have been used to improve the low brain drug bioavailability (Terstappen et al., 2021).

As for the skin, it is the largest (1.8 m^2 and 16 % of the total body mass) and most exposed organ of the body. Therefore, it serves as the first defence against external factors such as UV radiation, microorganisms and chemicals. Thus, the skin is one of the most difficult barriers that drugs and DDS have to cross. (Krishnan and Mitragotri, 2020).

Skin is composed of two primary layers, epidermis and dermis, and other sublayers (Figure 5), which have different components such as ephitelium, mesenchymal, glandular, and neurovascular.

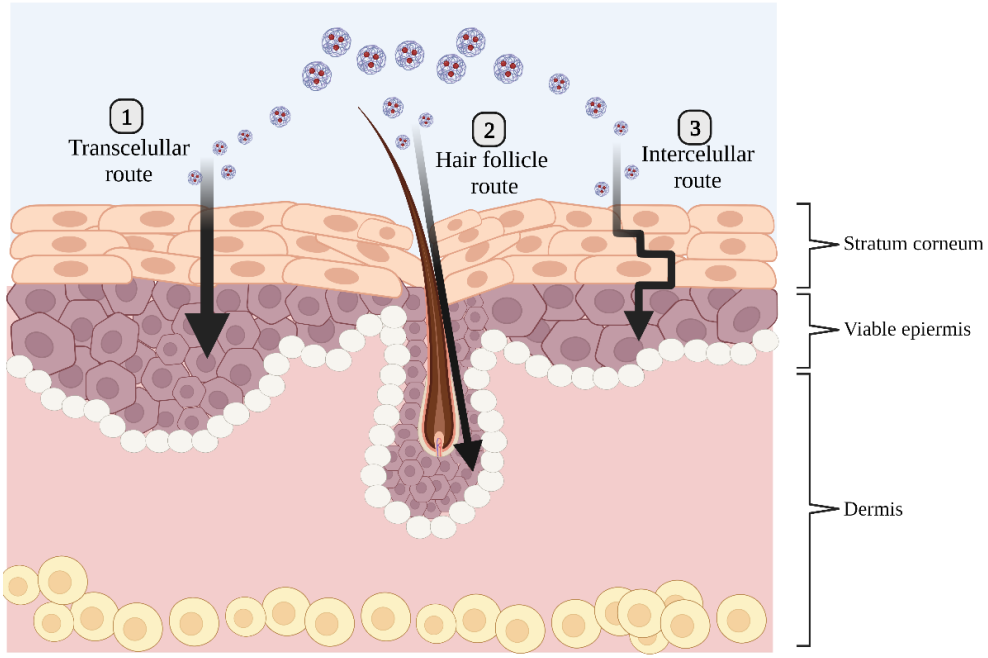

Figure 5. *Illustration of skin structure and the three possible penetration pathways (1, transcellular route; 2, hair follicle route; 3, intercellular route) for applied active ingredient into and through skin.*

- Stratum corneum is the most superficial layer of the epidermis and therefore of the entire skin. This layer has a thickness of 10-20 µm and are made up of corneocytes (terminal differenced keratinocytes composed of cross-linked keratin) surrounded by lipid (ceramides, cholesterol, and free fatty acids) organized in lamellar crystalline bilayers. Corneocytes together lipids form a network that absorb water and control the penetration of molecules through the skin (Heisig et al., 1996).

- Viable epidermis beneath stratum corneum is 50-100 µm thick, and are composed of several cell types: keratinocytes at different maturation stages, melanocytes, Merkel cells, and Langerhans cells. In addition, in this layer is where most dermatological disorders occur.

- The next layer is the dermis which contains collagen, elastin, lymphatic vessels, sensory nerves, sebaceous glands, and hair follicles. This layer has a thickness of 0.1-0.4 cm and its main role is to provide nutrition to the epidermis and act as a structural support for the entire skin (Krishnan and Mitragotri, 2020; Tiwari et al., 2022).

2.2. Applications of nanoencapsulation systems

The study of NPs and DDS has raised the interest in applying them in different fields like biomedicine, cosmetic, agriculture, and food industry (Figure 6).

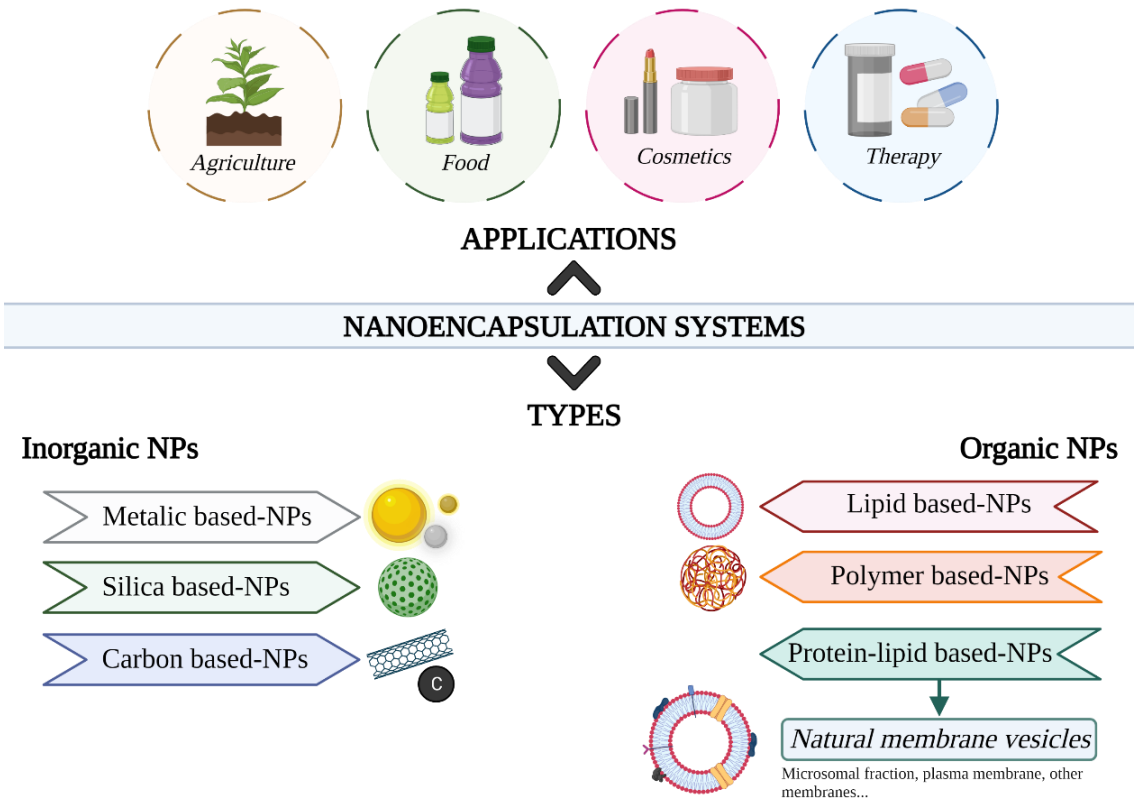

Figure 6. *Main applications of nanoencapsulation systems and classification.*

✓ Biomedicine

Advances in nanotechnology have allowed the development of useful recourses in different areas of medicine. Biosensors composed of gold or iron-based NPs have been developed to improve disease diagnosis (Martina et al., 2005). Regarding therapy, NPs have great relevance in tissue regeneration (Heo et al., 2014) and treatments against cancer by hyperthermia (Alonso et al., 2016). Although the application based on NPs most developed in biomedicine is the drug administration (small molecule drugs, proteins, and DNA/RNA). Thus, using NPs have allowed the control of administration pathways, toxicity, and drug dispersion in the organism (Mitchell et al., 2021).

✓ Cosmetic

Nanotechnology has been incorporated into the cosmetic formulations, and nanoscale size ingredients have been used in cosmetic products for dermatological, dental, and hair care applications (Aziz et al., 2019).

✓ Food

The use of NPs in the food industry is focused on enhancing the nutritional value, sensory response, and shelf life of food products. Besides, encapsulation of active compounds (nutraceuticals or preservatives) used in food increase their bioavailability and stability, and protect them from environmental degradation agents (Assadpour and Mahdi Jafari, 2019).

✓ Agriculture

Although compared to the medical area, the use of NPs in agriculture is less developed, promising results are being achieved in recent years (Rios et al., 2019; Rios et al., 2020). The main objectives for NPs in agriculture are increasing the crop production and reducing the environmental impact of extensive crops by diminishing evaporation and leaching of harmful substances such as of herbicides, fungicides, and insecticides (Duhan et al., 2017). Some potential application of NPs in agriculture are delivery fertilizers, micronutrients supply, nanofungicides, nanoherbicides and biosensors. The last is very promising since could help in the efficient use of essential resources in agriculture like nutrients, water or agrochemicals (Singh, 2021).

2.2.1. Nanoencapsulation in treatment of skin disorders

Skin disorders could be divided in different categories depending on the severity. On the one hand, less severe skin disorders can be treated from a cosmetic approach. Cosmetics has been defined as "*particles intended to be applied onto human bodies or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance*" (FDA, 2016). In addition, between pharmaceuticals and cosmetics are cosmeceuticals, which is a niche where the products have bioactive compounds to treat various disorders such as skin dryness, skin aging, hair damage, pigmentation, acne, broken blood vessels, etc. Thus is,

cosmeceuticals have a dual purpose: (1) providing esthetical effects, and (2) treating dermatological disorders.

In this area, the use of NPs has three functions, acting as nanocarriers, active substances, or modifiers of the characteristics of the final products (appearance or rheology). Nanoencapsulation provides to cosmetic formulations different improved characteristics such as deeper skin penetration, major UV protection, longer-lasting effects, enhanced anti-aging effect, or increased colour (Aziz et al., 2019).

On the other hand, acute disorders such as diseases with an inflammatory component (psoriasis, atopic dermatitis, or rosacea) or different skin cancer types, which are treated from a medical approach (Lee et al., 2021). Skin cancers are one of the most extended cancers in human, particularly in the white population, with over a million cases detected each year (Leiter et al., 2020). Generally, two types of skin cancers can be described, melanoma skin cancer (MSC) that originate from melanocytes and non-melanoma skin cancer (NMSC) that originate from epidermal-derived cells (Simões et al., 2015). Malignant melanoma is deadliest skin cancer and its incidence has increased over the past years. Different changes in lifestyle are related to this increase, but mainly an increased exposure to UV radiation has been observed as one of the main responsible factors (Leiter et al., 2020). The future in the treatment of these disorders is focused on achieving less side effects and doing the treatment in a local form. Nanoencapsulation is therefore at the centre of the development of new treatments.

2.3. Administration routes

Taking into account the final applications and the biological barriers that the DDS have to cross to reach the target site, different routes of administration can be proposed, which are listed in Table 1.

Table 1. *Different administration routes of drug delivery systems.*

Name of route	**Way of administration**
Oral	Drug is administered through mouth
Parenteral	
o Intramuscular	Drug is administered into the skeletal muscles
o Subcutaneous	Drug is given under the skin
o Intravenous	Drug is directly given in the veins
Transdermal	Drug is administered in the dermal region
Topical	Drug is applied over the skin

Administration of drugs, both free or encapsulated in DDS, through skin have some advantages over other routes of administration. Among the advantages are the following:

- ✓ Systemic side effects are avoided and are generally few and localized.
- ✓ Elimination of the risk of digestion/degradation of drugs in the gastrointestinal tract.
- ✓ A rapid metabolism, enzymatic degradation and clearance of blood circulation are prevented.
- ✓ Easy application and suitable for self-medication.
- ✓ Drug delivery into a specific site.

After transdermal or topical delivery of drugs, there are three possible pathways to reach the target, which are transcellular, intercellular, and hair follicles (Figure 5) (Aziz et al., 2019).

- Transcellular penetration involving a delivery of drugs through layers of lipids and corneocytes to de living cells.
- The hair follicles path serves as penetration medium since follicles have a dense network of blood capillaries. In addition, they act as reservoir of drugs applied onto skin.
- Finally, the intercellular pathway occurs when active compounds pass through stratum corneum via the lipid layers that are surrounded by keratinized cells.

Once described the possible pathways of drug administration through skin and the existing difficulties for drugs to cross the SC, various methods have been proposed to enhance the skin permeability such as chemical permeation enhancement, permeation enhancement by physical means, or biomolecule-mediated enhancement (i.e. peptide cell penetration enhancer). Although the most promising and less invasive method is the enhancement of drug delivery by nanoencapsulation systems (Yang et al., 2017).

2.4. Types of nanoencapsulation systems

A general classification of NPs or DDS is based on the nature of the materials. Hence, NPs are divided into organic and inorganic, and within each type, there are different sub-classifications that are explained below (Figure 6)**.**

2.4.1. Inorganic nanocarriers

There are different types of inorganic NPs such as silica-based NPs, carbon-based NPs and the most popular type, metallic NPs. Inorganic nanocarriers have some disadvantages, as toxicity derived from the synthesis process and the generation of reactive oxygen species (ROS) in the target cells of the final application (Soenen et al., 2011). In this sense, obtaining nanocarriers from natural sources, which would be more environmentally and economically friendly, is the path that is currently being promoted and sought. In the last years, green synthesis procedures have been developed to get metallic NPs from biological resources such as plants, fungi, bacteria, yeast, and human cells (Mohanpuria et al., 2008). Recently, an eco-friendly and low-cost method to synthetize silver NPs from fresh leaves of *Acacia melanoxylon* has been described to use them as dopamine and H_2O_2 sensor (Shashanka and Kumara Swamy, 2020). One of the most studied metallic based-NPs are gold-NPs, since they have a lot of potential applications, especially in medicine field: destruction of tumours by localized hyperthermia, radiotherapy for cancer or in conjugation with proteins are used as inmunosensors (Elahi et al., 2018). Besides silver or gold NPs, other commonly synthesized metallic NPs are iron, iron oxide, zinc, titanium, aluminium, cadmium, and lead.

These type of NPs have potential uses in biotechnology, specifically in preconcentration of the targeted analytes, drug/gene delivery, diagnostic imaging, and biosensors for diagnosis (Rout et al., 2018). Besides, metallic NPs can be modified with chemical groups which facilitate the conjugation with ligands, drugs, or antibodies.

Although the possibility of obtaining these NPs through eco-friendly processes has promoted their production and use, there is some controversy with *in vitro* and *in vivo* toxicities of these elements (Rout et al., 2018). Therefore, alternatives to inorganic NPs are necessary.

2.4.2. Organic nanocarriers

Organic nanocarriers are particles composed of lipids, proteins or polymers in a diameter from 10 to 1000 nm. Currently there is an increasing interest in developing therapies or finding drugs from natural sources, and this is being seen in the same way in the area of nanotechnology and specifically nanocarriers. Organic nanocarriers stand out for their high biocompatibility, their great capacity of encapsulation as well as for their versatility to encapsulate both compounds of hydrophilic and lipophilic character. Organic NPs include liposomes, micelles, protein/peptide-based NPs, lipid-protein NPs (proteoliposomes), dendrimers, microemulsions and niosomes.

2.4.2.1. Liposomes and proteoliposomes

The most popular organic NPs are liposomes, which were discovered in 1965 (Bangham et al., 1965). First, liposomes were described as good models for the *in vitro* study of biological membranes. Later, considering the properties of liposomes, possible uses of these lipid vesicles in biotechnological applications began to be considered, for example as drug delivery and encapsulation systems. Liposomes are spherical vesicles with an aqueous core (hydrophilic character) surrounded by one or more lipid bilayers (hydrophobic character) of phospholipids, which are formed by two hydrophobic hydrocarbon chain of fatty

acids join to a hydrophilic polar head, which are in contact to aqueous core and outside (membrane surface).

Regarding types of liposomes, these are classified based on different criteria, but the most common classification is referred to the size and the number of bilayer (Figure 7):

- Small unilamellar vesicles (SUV): liposomes made up of a single bilayer with a diameter of 20 to 80 nm.
- Large unilamellar vesicles (LUV): liposomes made up of a single bilayer with a diameter of 80 to 1000 nm.
- Multilamellar vesicles (MLV): liposomes made up of two or more bilayers with a diameter of 400 nm to several µm.

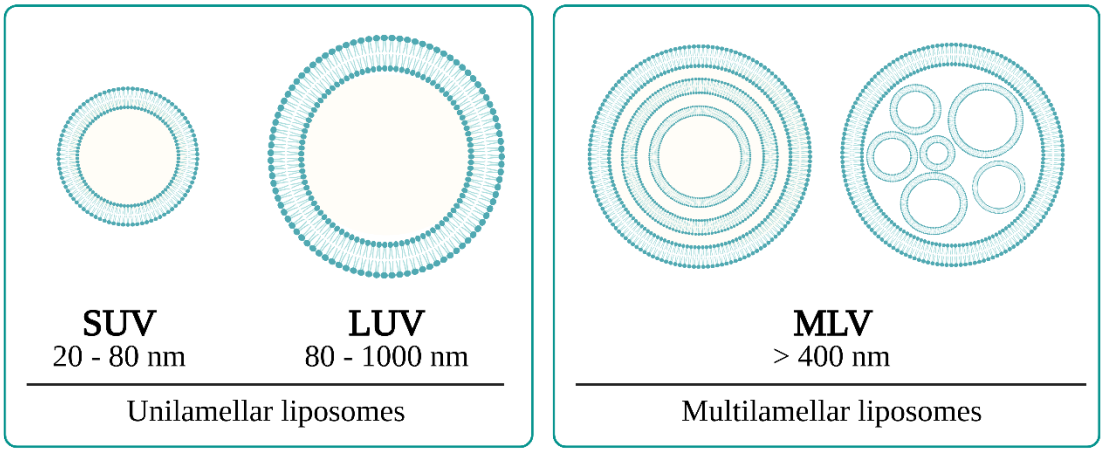

Figure 7. *Liposomes classification according to the size and the number of bilayers.*

Because liposomes are consider amphipathic structures, since they have both lipid and aqueous phases, have the advantage of encapsulating both lipophilic and water-soluble compounds; hydrophilic molecules are encapsulated in the aqueous core, while lipophilic compounds are entrapped into lipid bilayer (Carugo et al., 2016). This type of vesicle was the first DDS used in medical and cosmetic applications with the main objective of improving the *in vitro* and *in vivo* properties of encapsulated substances (protein, drug, RNA, DNA, or bioactive extract) (Lasic, 1997).

The similarity of liposomes with cell membranes due to their characteristic bilayer structure gives this type of nanocarrier great compatibility and permeability to cross biological barriers (skin, mucosal membranes, BBB or enterocytes), interact with target cells and delivery encapsulated compounds. Interaction between liposomes and eukaryotic cells is produced through different mechanisms: fusion, endocytosis (clathrin or caveolae-mediated), macropynocitosis, phagocytosis, absorption, or lipid exchange (Gonzalez Gomez and Hosseinidoust, 2020).

The use of liposomes to encapsulate and transport bioactive molecules have different **advantages** (Allahou et al., 2021), which are summarized below:

- ✓ Liposomes can carry both hydrophilic and lipophilic substances, as well as negatively and positively charged molecules.
- ✓ Protection and stabilization of encapsulated substances from hostile environment regarding their bioactivity and beneficial properties, extending their half-life *in vitro* during storage, and *in vivo* at the final organism.
- ✓ Provide direct contact of drug with target cell and allow a site-specific drug delivery.
- ✓ Liposomes can provide a sustained drug release.
- ✓ Liposomes are biodegradable and biocompatible.

Even though liposomes are the most used system to transport active ingredients, they have some **limitations** (Allahou et al., 2021), which are summarized in:

- x The production of liposomes is economically expensive.
- x Phospholipids are more instable than other membrane components because they are sensible to oxidation and hydrolysis-like reactions, which reduce the half-life.
- x There are some leakage and fusion of encapsulated molecules, and aggregation phenomena are possible.
- x Liposomes can be less stables.

Therefore, new formulations to use as DDS are being investigated. To modify conventional liposomes with the objective of improve their characteristics is very common, for example add polyethylene glycol (PEG), which is one of additives most used for modifying liposome surface and obtain some desirable properties as prolonged circulation times or better *in vitro* target binding (Mitchell et al., 2021). Liposomes can be also modified by adding proteins, known as proteoliposomes. This type of vesicles has gained relevance not only in studies about interactions between lipids with proteins but as systems for use in biotechnological applications as nanocarriers, as well as in of nanosensors and vaccines area (Tenchov et al., 2021; Estephan et al., 2022).

Proteoliposomes have the advantages of liposomes, but also the added value of proteoliposomes is the presence of functional proteins, which contribute to improve some properties such as an efficient intracellular delivery, increased targeting, or improved circulation (Lu et al., 2018). In addition, the protein-lipid interactions simulating the native environment membranes provide an additional stability to vesicle system (Martínez Ballesta et al., 2016; Seneviratne et al., 2018). However, proteoliposomes have the same disadvantage of liposomes and others such as standardized method is required for each specific proteins, and it is necessary to verify the orientation and the functionality of proteins (Ciancaglini et al., 2012).

2.4.2.2. Natural membrane vesicles

In addition to liposomes and proteoliposomes *in vitro* synthetized, proteoliposome-like vesicles can be obtained from natural sources such as mammals (cell cultures), plants, yeasts, or bacteria. These vesicles are another alternative to use as DDS in therapy or cosmetic for delivering the drug to specific cells and tissues (Wang et al., 2013), and as phospholipid structures can be considered analogous to liposomes. In addition, since these vesicles are natural and their origin is cellular are more biocompatible and safer (Nemati et al., 2022).

Natural membrane vesicles like exosomes from different sources are structurally composed of lipids and proteins, and several components accumulated

randomly from original cells such as bioactive compounds, nucleic acids, or proteins (Figure 8) (Akuma et al., 2019).

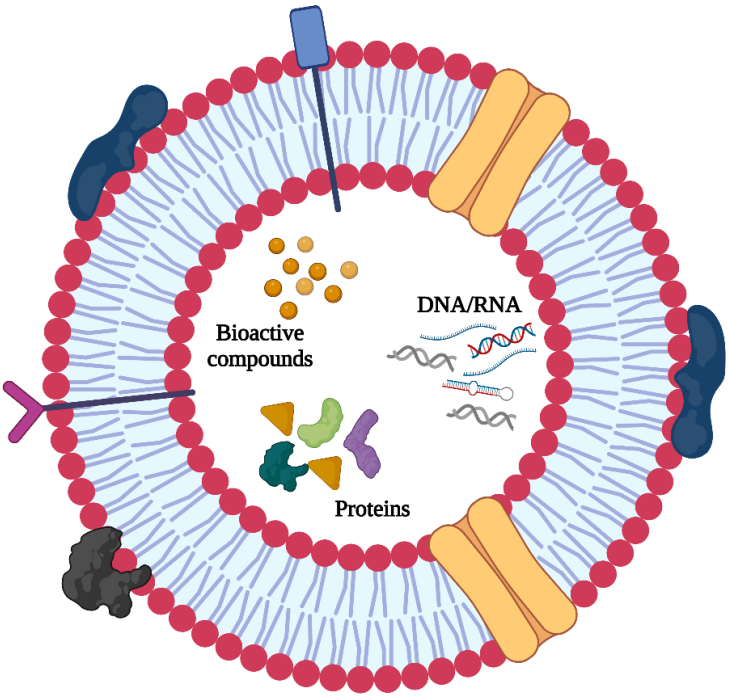

Figure 8. *Representative structure of natural membrane vesicles composed of integral proteins and lipids containing bioactive compounds, proteins and nucleic acids.*

3. Plant-derived membrane vesicles

Plant-derived membrane vesicles will stand out among the natural membrane vesicles, since they are the main topic of this PhD thesis.

Membrane vesicles purified from non-toxic plants are proposed as new DDS that aim to eliminate the problems associated with other existing DDS. In addition to the composition and natural origin, they represent an opportunity to obtain vesicles in bulk from economical plant sources. Different plant species have been used as source of membrane vesicles such as ginger, grapefruit, grape, lemon, apple, strawberry, broccoli, etc. (Wang et al., 2022). So, in recent years, many efforts have been made on research applications of plant-derived vesicles, making great advances in this regard (Figure 9).

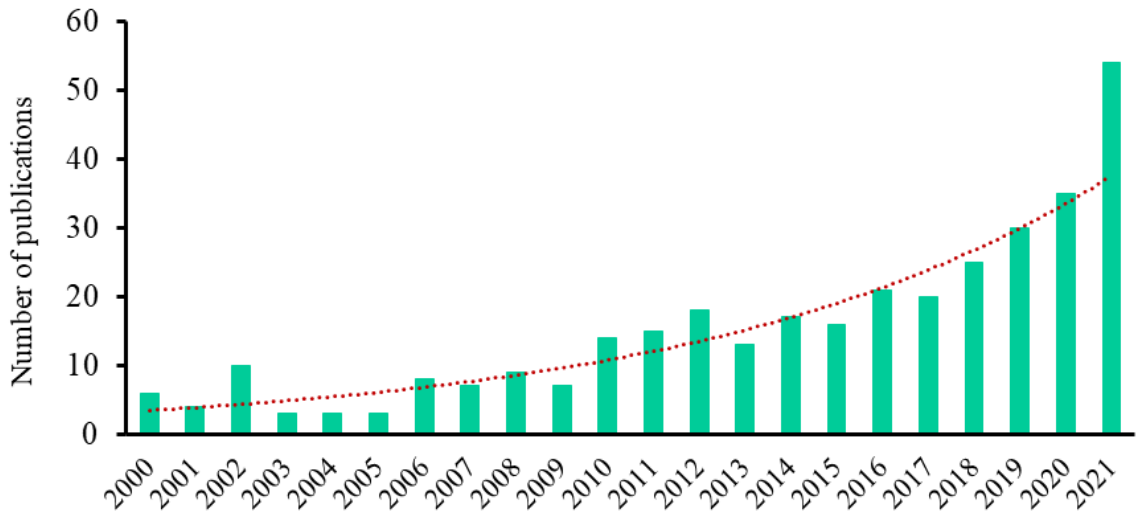

Figure 9. *Number of publications about "plant-derived vesicles" and their "biotechnological applications" in the last 20 years.*

3.1. Composition and types of plant-derived membrane vesicles

Plant-derived membrane vesicles can be characterized by their size, morphology, and biochemical composition. The structural and physicochemical characteristics of these membrane vesicles are different depending on source material, growth condition of source plants, and type of purified membrane (microsomal fraction (MF), plasma membrane (PM), membranes from different organelles, etc.) (Figure 10). These membrane vesicles are biological membranes and therefore are composed mainly of a complex mixture of lipids and membrane proteins (superficial and integral), in addition to nucleic acids and different metabolites in the interior (Figure 8).

Regarding size, they are generally larger than animal-derived membrane vesicles. Plant membrane vesicles have a mean diameter between 30 and 600 nm, and a negative Z-potential above -20 mV, with high stability (Ju et al., 2013; Wang et al., 2014; Fujita et al., 2018). Following the previous criteria, these membrane vesicles could be similar to LUV and MLV. Thus, this type of vesicles offers high versatility, and it is possible to get vesicles of different compositions, which can provide specific properties to the vesicles and adapt them to several applications in therapy, cosmetics, food, or agriculture.

These vesicles have some advantages with respect to the DDS described above.

- ✓ Plant-derived vesicles have a much lower cost than synthetic liposomes or proteoliposomes, since a bulk extraction from economical plant sources is possible.
- ✓ It is possible to get plant-derived vesicles from agriculture by-products (leaf or roots), which would help to establish a circular economy, in which the maximum use of materials and a reduction in waste are sought.
- ✓ These vesicles are biodegradable and biocompatible, a similarity in lipid-protein composition to mammalian exosomes has been stabilised for some plant membrane vesicles and therefore comprising low immunity (Ju et al., 2013; Vaishnav et al., 2013).
- ✓ These vesicles can be modified and loaded with molecules and agents as bioactive compounds, drugs, RNAs…

3.2. Isolation and characterization methods of plant membrane vesicles

In procedures to isolate membrane vesicles is essential to separate accurately them from plant tissues. Methods to isolate animal-derived exosomes are highly standardized, but due to differences with plant-derived vesicles, the protocols have to be adapted. In the last years, different methods have been developed and now there is enough variety: differential ultracentrifugation, gradient density centrifugation, PEG precipitation, size exclusion chromatography (SEC), electrophoresis, and ultrafiltration membrane separation.

- ***Differential ultracentrifugation***

Ultracentrifugation is the most used procedure to isolate plant-derived membrane vesicles. This method consists of two main steps after crushing the raw material: one centrifugation at low speed (about 10,000 g) for removing high debris from plant tissues, where supernatant with vesicles is retained, and other centrifugation to precipitate membrane vesicles at high centrifugal force (about 100,000 g). Ultracentrifugation have the advantages of vesicles obtained have a high purity and the operations are simple. On the other hand, the complete process

imply long time and a high cost, since specialized equipment is needed (Yang et al., 2020).

- ***Gradient density centrifugation***

Gradient centrifugation is a procedure that is usually coupled after differential ultracentrifugation to obtain vesicles with higher purity. Sucrose at different concentrations is normally used to generate the gradients. And, additional time to process is required (about 1 to 5 h) (Yang et al., 2020).

- ***PEG precipitation***

PEG precipitation method was proposed as ultracentrifugation requires high-cost ultracentrifuges, although this method involves a simple and economical operation, the yield is low and the isolated vesicles have low purity (Wang et al., 2022).

- ***Size exclusion chromatography***

This chromatography method is less used than method described above due to a heavy workload and a long time are required. Nevertheless, it has been reported that vesicles with less contamination with non-vesicular proteins and other molecules are obtained compared to ultracentrifugation procedure (Gardiner et al., 2016).

- ***Electrophoresis***

Electrophoresis followed by dialysis seems to be a good option without many drawbacks. After separation under the action of the electric field and dialysis bag, vesicles obtained have similar characteristic to those obtained by ultracentrifugation (Yang et al., 2020).

- ***Ultrafiltration***

Ultrafiltration has exhibited great potential to use in combination with other methods to obtain vesicles with high purity. Besides, it is fast and does not require special equipment (Li et al., 2017).

All these methods present advantages and disadvantages. In different reports, it has been shown that the most appropriate to purify membrane vesicles with suitable properties is to employ different methods in a combined way (Gardiner et al., 2016). Regarding physical characterization methods, dynamic light scattering

(DLS) technology and transmission electron microscopy (TEM) are widely used for determining membrane characteristics. After the purification of membrane vesicles, a physical characterization is essential to determine the stability and authenticity of particles. In this sense, the following parameters are always determined:

- Size and polydispersity index
- Z-potential
- Morphology

3.3. Bioactivity of plant membrane vesicles and their application in human disorders

Plants have been widely studied for many years due to their beneficial properties for human health. So, membrane vesicles obtained from plants have been shown to retain part of these properties. Different works have demonstrated the biological activity of edible plant-derived vesicles:

- ✓ Anti-inflammatory effect
- ✓ Anticancer effect
- ✓ Antibacterial and antifungal effects
- ✓ Antioxidant effect

These effects are produced through different ways, such as interaction with the intestinal microflora, gene regulation, gene silencing, acting on macrophages, induction of apoptosis, etc. (Wang et al., 2014; Sundaram et al., 2019; Zhang et al., 2021).

As regard possible applications of plant membrane vesicles, on the one hand membrane vesicles can act as drug or bioactive compounds (Figure 10). Although research in this area is recent, they have already been investigated to treat different diseases. For example, vesicles from grapefruit (Wang et al., 2013; Wang et al., 2014) and ginger (Teng et al., 2018) have been tested to treat colitis.

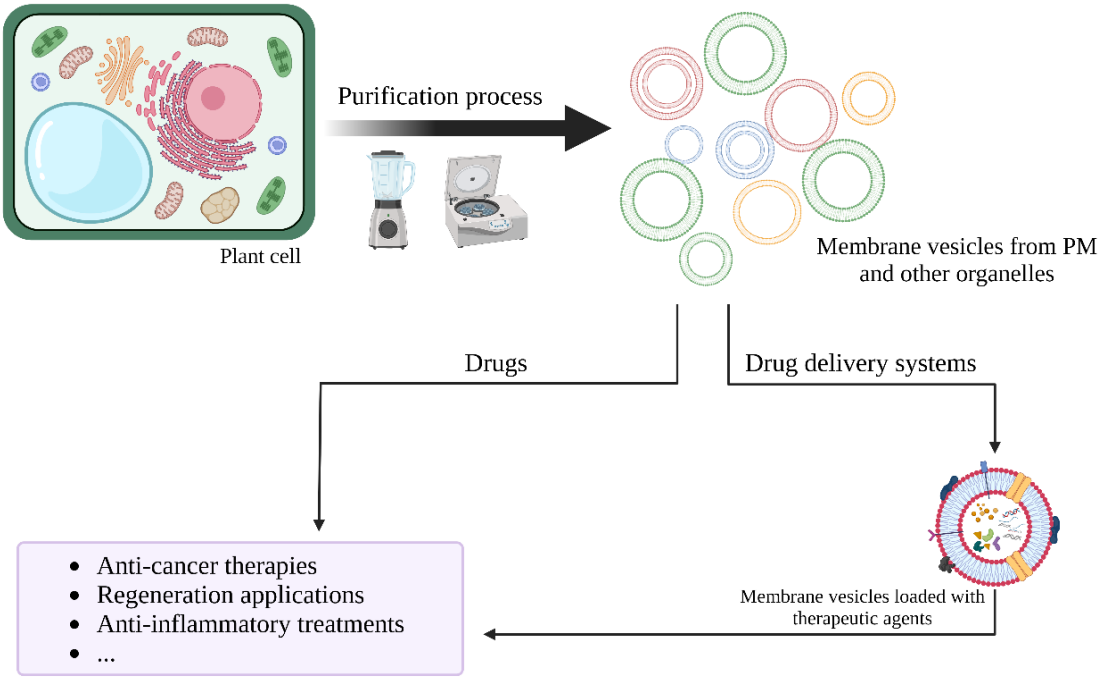

Figure 10. *Schematic representation about purification of plant vesicles from different membrane classes and possible applications.*

Colitis is a disease that causes pathological changes in the colon by immune factors and has an important inflammatory factor (Ng et al., 2017). So, it has been shown membrane vesicles from edible plants have an important role both in prevention and treatment of this disease and different action mechanisms have been described (Wang et al., 2022):

- Regulation of intestinal macrophages.
- Promoting the proliferation of intestinal stem cells.
- Regulation of intestinal immune environment homeostasis.
- Modifying intestinal microflora.

On the other hand, membrane vesicles, as indicated above, can be used as DDS for the treatment of diseases or in cosmetic applications (Figure 10). It has been described that plant-derived membrane vesicles not only have good absorption in the intestine, but also some of them can enter the BBB and thus can deliver small molecular drug such as proteins, siRNA, DNA expression vectors or chemotherapeutic drugs (Wang et al., 2022).

4. Broccoli-derived membrane vesicles: background

Cell membranes at protein and lipid level have been widely studied by our group in broccoli plants (López-Pérez et al., 2009; Casado-Vela et al., 2010; Chalbi et al., 2015). Biochemical changes in PM of *Brassica* roots and leaves related with the resistance to abiotic stress were reported and the modification of lipid composition of PM was linked to regulation of permeability and protein function (Chalbi et al., 2015). PM vesicles from *Brassica oleracea* were described by their higher thermodynamic stability compared to PM from other plants such as *Brassica napus* or *Cakile maritima* (Chalbi et al., 2015). Thus, the degree of saturation of fatty acids, sterol composition, protein/lipid ratio, protein composition and functionality were shown as important aspect to take into account for the design of proteoliposomes in relation to the specific application and target tissue. Therefore, the modulation of these parameters to obtain custom natural proteoliposomes is a future field of research. To obtain natural proteoliposomes with specific characteristics is possible by purification of vesicles from different membrane fractions such as MF, PM, or even "lipids raft" (detergent resistant membranes, DRMs) (Martínez Ballesta et al., 2016; Martínez Ballesta et al., 2018; Yepes-Molina et al., 2020) (Figure 11).

Besides, different plant tissues can also be employed to obtain vesicles, which is an important factor since an economic point of view. Since, the use of agricultural by-products, which have no value, representing an economic benefit (Domínguez-Perles et al., 2010). Regarding raw material, in real field conditions, obtaining roots of crops would entail extra work that would have to be taken into account in the final costs and assess whether it is profitable. Thus, isolating membrane vesicles from leaves is easier and cheaper. On the other hand, regarding the different membrane fractions, the most economical and industrially scalable would be MF vesicles, although other membrane fractions would have to be considered depending on the final use.

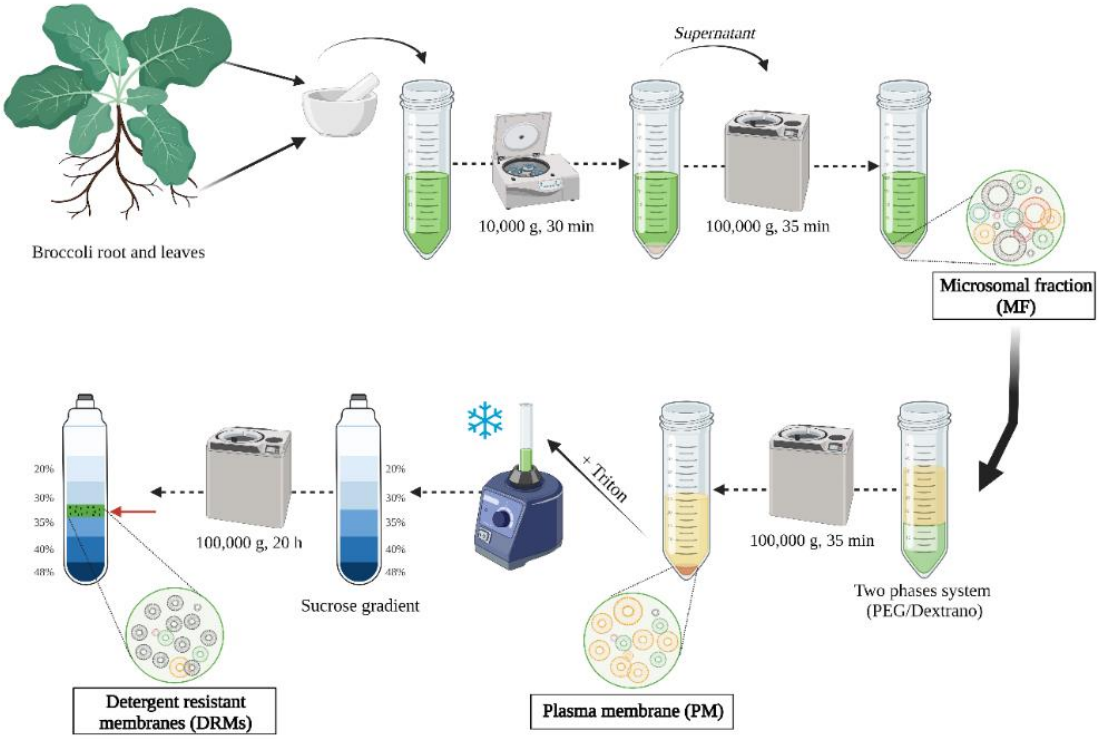

Figure 11. *Schematic diagram of the protocol for isolating vesicles from different membrane fractions: microsomal fraction (MF), plasma membrane (PM), and detergent resistant membranes (DRMs) or "lipid raft". Protocol according Yepes-Molina et al., (2020).*

All membrane fraction mentioned above form vesicular structures of different size (Figure 12), which plays a key role and determining the properties and performance in biotechnological applications such as entrapment efficiency of compounds, *in vitro* stability, interaction with target cells or penetration through biological barrier as skin (Yu et al., 2020; Dolai et al., 2021). Besides, due to these vesicles are lipid-protein membranes, osmotic water permeability (P_f) is determined in order to determine integrity and functionality of membrane vesicles. In this sense, it was reported that PM vesicles had the highest P_f, followed by the MF and after the DRMs vesicles (Figure 12) (Martínez Ballesta et al., 2018; Yepes-Molina et al., 2020). These characteristics affect to vesicle surface and therefore influence the release of the encapsulated compound and the interaction with the surface of target cells. Recently, vesicle membrane fluidity has been shown to be a key parameter for selectively targeting cancer cells (Bompard et al., 2020), since

fluid liposomes interacting more with cancer cells compared to more rigid liposomes.

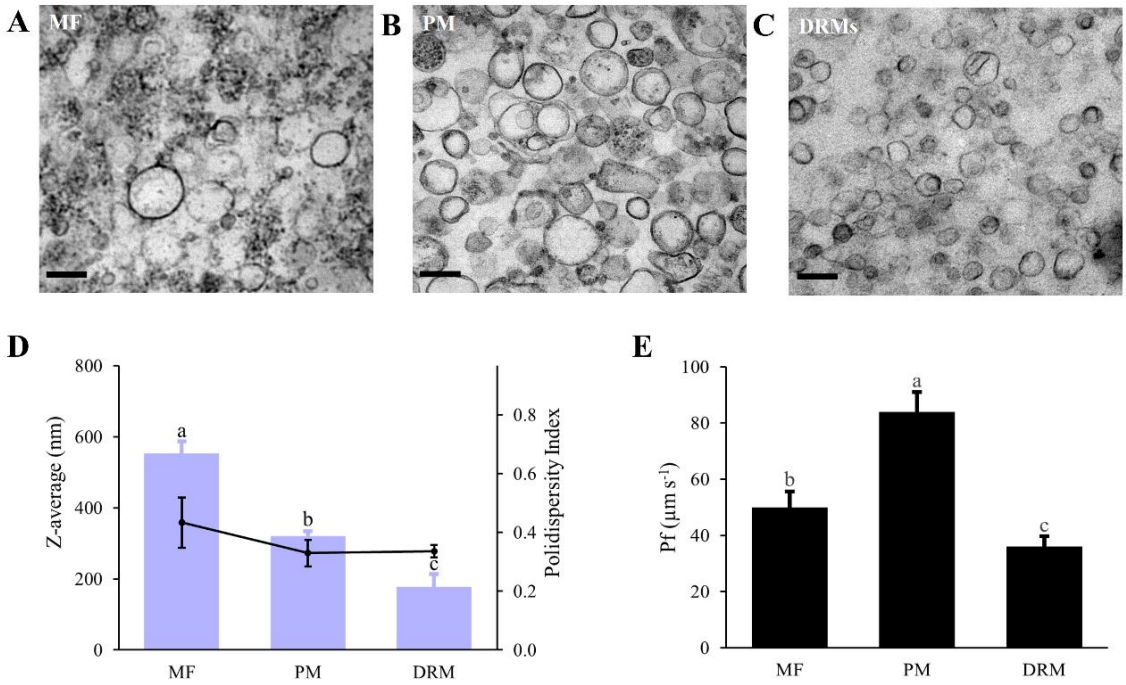

Figure 12. *Physicochemical characterization of membrane vesicles**.*** *Transmission electron microscopy images of microsomal fraction (MF) (A), plasma membrane (PM) (B) and detergent resistant membrane (DRM) vesicles purified from broccoli (C). Scale bar = 500 nm. Z-average (nm) and polydispersity index of MF, PM and DRMs (D). Osmotic water permeability (P_f, μm s^{-1}) of MF, PM and DRMs. Data are means ± SE (n = 3-5). Distinct letters represent significant differences based on one-way ANOVA followed by Tukey HSD test (P < 0.05). Data adapted from Martínez Ballesta et al., (2018) and Yepes-Molina et al., (2020).*

Regarding proteins content in membranes, AQPs were revealed as key in the stabilization of a bioactive compound (glucosinolate glucoraphanin) encapsulated in membrane vesicles from broccoli (Martínez Ballesta et al., 2016). Besides, water purification filters were made with AQP-reconstituted proteoliposomes, and results obtained from nanofiltration assays showed that these vesicles were stable under stirring shear and pressure (Sun et al., 2013). So, AQPs not only facility water transport, but also have a role in membrane stabilization *in vitro*. AQPs are key in plant adaptation to changing environmental conditions by modifying the permeability of cell membranes, and in broccoli an increase in the level of AQPs transcripts under salinity stress conditions has been reported (Muries et al., 2011).

In this context, membrane vesicles isolated from *Brassica* species, which under certain growing conditions are enriched in AQPs, have been obtained through a patented method (PCT/ES2012/070366) (Carvajal et al., 2011) for its use in biotechnological applications.

4.1. Aquaporins

4.1.1. Structure, selectivity and location

AQPs are membrane proteins belonging to the mayor intrinsic protein superfamily (MIP). These proteins appear in all organisms and their principal role is to act as water channels, although small neutral solutes or gases can be also transported (Maurel et al., 1993; King et al., 2004; Finn and Cerdá, 2015). These proteins are assembled in tetramers, where each monomer have a molecular weight of 25 – 30 kDa. Monomers consist of six transmembrane α-helices (H1-H6) connected by five loops (A-E) with N and C termini located on the cytoplasmic side of the membrane (Murata et al., 2000; Törnroth-Horsefield et al., 2006). This structure forms a specific pore that results from two selectivity filters, which allow exclusion of molecules passing through the pores: (1) The first one is a pair of conserved domains formed by three residues Asn-Pro-Ala (NPA), (2) and the second filter is the so-called ar/R (aromatic/arginine) region formed by two aromatics residues and one arginine (Murata et al., 2000) (Figure 13).

Plant AQPs are usually classified in seven subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), unknown intrinsic proteins (XIPs), hybrid intrinsic proteins (HIPs) and GLpF-like intrinsic proteins (GIPs) (Danielson and Johanson, 2008). Unlike this diversity found in plants, mammals possess only 12 to 15 isoforms include in 12 subfamilies (AQP0-AQP12) (Finn and Cerdá, 2015). Comparing both animal and plant AQPs, phylogenetic studies revealed certain proximity: AQP1 and PIP, AQP8 and TIP, AQP3 and NIP, and AQP11 and SIP. This opens up a new paradigm of vertical transfer of four ancestral MIP subfamilies (Soto et al., 2012) and supports recent studies about cross-

reactions between plant and animal proteins and membranes (Ju et al., 2013; Vaishnav et al., 2013).

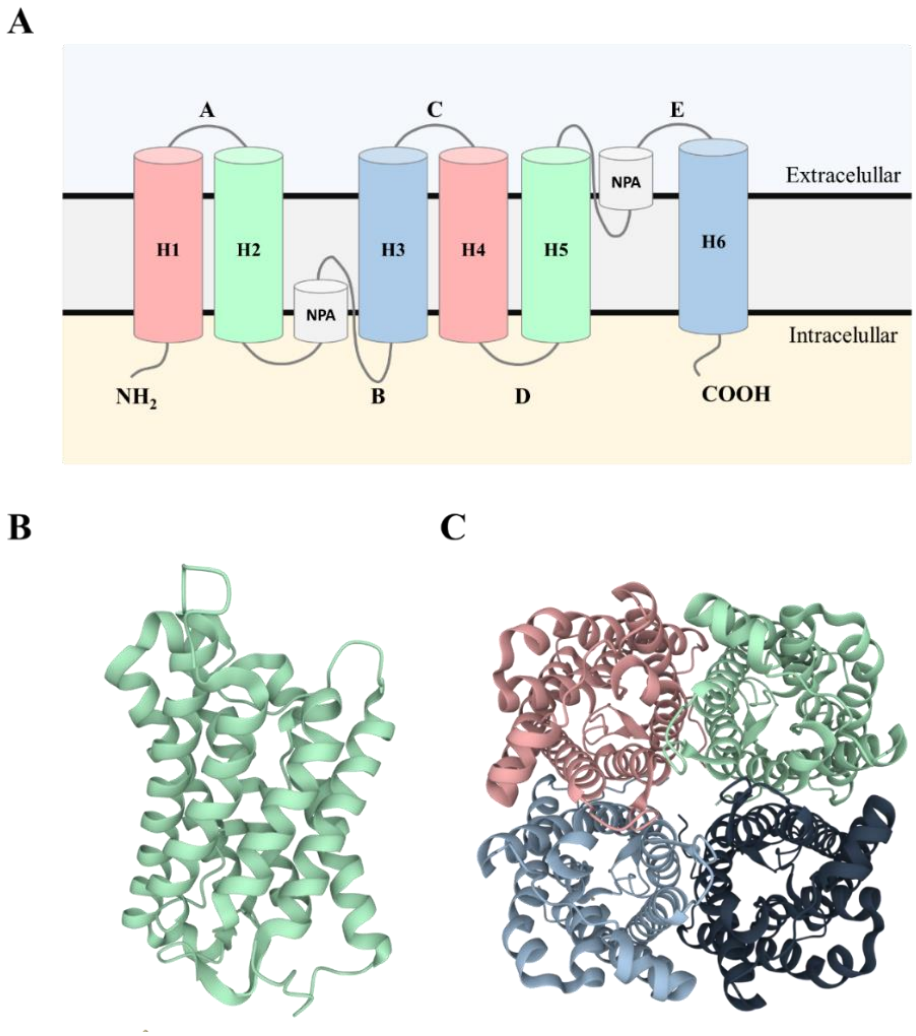

Figure 13. *Structure of AQP monomer inserted in bilayer membrane with six α-helices (H1-H6) connected by five loops (A-E) (A). 3D structure of a monomer (B) and tetramer (C) of Spinacea oleracea SoPIP2;1 (structures obtained and modified from Protein Data Bank – PDB (Törnroth-Horsefield et al., 2006)).*

AQPs are important for water homeostasis in cells, organs and organisms, both in mammals and plants. In plants, AQPs are essential in the transport of water across plant, specifically short-distance transport via the transcellular route, which requires water to cross cell membranes. Thus, AQPs act in the latter case by modulating membrane permeability (Maurel et al., 2015). Regarding mammals, water transport plays a function in cell migration, which could be key in the development of numerous types of cancer. Thus, the focus is on AQPs to find

targets for drug development strategies (Wang et al., 2015), and a cross research between plants and mammals AQPs could be relevant or help in this purpose.

4.1.2. Aquaporins in membranes

Eukaryotic cells and most organelles are surrounding by a semipermeable bilayer membrane, which in case PM is a selective barrier between cells and the outer environment. Biological membranes consist of lipids and proteins that establish chemical and physical communication both among cells and intracellular compartments. Membrane proteins can be transmembrane/integral, peripheral or lipid-anchored (Guidotti, 1972), and they respond to changes in the local environment acting as receptor, transporter or channels, as would be the case of AQPs. The response to external stimuli entails an activity regulation of the membrane proteins.

AQPs activity is modulated by various regulation processes in order to control the water and solute homeostasis. Permeability through AQPs can be modulated by opening or closing (gating) or regulating membrane trafficking, since integral membrane proteins are synthetized in the endoplasmic reticulum (ER) and reach their final target membrane across the secretory pathway. In addition to the most studied regulatory mechanisms such as gene expression, posttranslational modifications, heterotetramerization, hormones, pH and cations (Ca^{2+}) or chemical agents (mercury or sodium azide) (Chaumont and Tyerman, 2014), in the modulation AQPs activity is also key the composition of the lipid environment, as well as the interaction with other proteins (Kai and Kaldenhoff, 2014).

4.1.2.1. Membrane nanodomains (“lipid rafts”)

Regarding distribution of proteins and lipids in membranes, new approaches have appeared against the classic “fluid mosaic” model (homogeneous and randomly distribution of membrane components) (Singer and Nicolson, 1972). The new proposed models are based on the presence in membranes of called “lipid raft”, which are nanodomains enriched in sterols/sphingolipids and in some proteins (Anderson and Jacobson, 2002). In plants, raft nanodomains are being

studied for 20 years (Peskan et al., 2000) and were related to detergent resistant membranes (DRMs). Heterogeneity of membrane suggests an important role of lipid composition in the activity of integral membrane proteins.

Protein composition in lipid raft is different from the rest of the membrane, and proteins together with lipids form signalling platforms involved in several biological functions: membrane trafficking, response against biotic and abiotic stresses, signal transduction, endocytosis, and cell-cell interactions (Tapken and Murphy, 2015). Regulation of these processes is possible because lipid raft membranes carry out a temporal and spatial organization of specific proteins, such as AQPs (Lefebvre et al., 2007; Minami et al., 2009; Belugin et al., 2011) establishing an optimal arrangement and suitable proportions. Influence of sterols and sphingolipids in distribution through PM and trafficking of PIP2;1 has been established (Li et al., 2011), and an essential role in the regulation of AQPs membrane functionality has been associated with lipids raft.

4.1.2.2. Mutual interactions between AQPs and lipids

Lipid-protein interactions underline the complexity of the biological membrane and therefore the physical properties of membranes (fluidity, permeability or viscosity) cannot just be related to lipid chemical composition. Thus, a role of membrane proteins may be considered. Regarding AQPs, a strong interrelation between membrane composition and AQPs activity have been reported in both plant and animal cells (López-Pérez et al., 2009; Tong et al., 2012). Sterols are related with water permeability and fluidity of membranes and they have a direct role on AQP functionality and trafficking across membranes. Lipid composition regulation is essential in the response to stress. Thus, sitosterol is very efficient in modulation of water permeability under salt stress and in addition, it is related to AQP functionality (López-Pérez et al., 2009; Basyuni et al., 2012). Furthermore, sterols can regulate the permeability of gases, not just water, across membranes by modulating AQP gas transport (Itel et al., 2012). Similar to sterols, phospholipids composition are linked to membrane properties and AQP activity (Carvajal et al., 1996; Martínez-Ballesta and Carvajal, 2016).

4.1.2.3. Mutual interactions between AQPs and other proteins

AQPs interact not only with each other but also with other proteins, which entails a regulation or post-translational modifications affecting gating and subcellular locations (Chevalier and Chaumont, 2015), and following this research line, about 400 proteins have been reported to interact with some PIPs isoforms (Bellati et al., 2016). For example, SNARE proteins (soluble N-ethylmale-imide-sensitive factor-attachment protein receptors) interact with PIPs in the trans-Golgi network to regulate the AQPs trafficking, their activity, and abundance (Hachez et al., 2014a); and a tryptophan-rich sensory protein (TSPO), which is induced under abiotic stresses, interacts with PIP2;7 to regulate their abundance by autophagy (Hachez et al., 2014b). All these interactions entail a modification of membranes water permeability related with AQP activity.

4.2. Role of aquaporins in the *in vitro* stability of membrane vesicles

4.2.1. Osmotic water permeability

The role of membrane proteins in vesicle stability, in particular AQPs, has been further explored by Martínez Ballesta et al., (2018), where a study with membranes from broccoli plants grown under both control and salt stress conditions were performed. Since an increase in AQPs amount in PM was showed previously in different broccoli cultivars under salt stress (Muries et al., 2013). The AQPs function stability was characterized by measurements of P_f over time by stopped-flow light scattering (Gerbeau et al., 2002), and obtained results showed a higher stability of NaCl PM vesicles (Figure 14A). Although, P_f measurements are correlated with the AQP activity, but not with the amount of AQPs, which could not be functional (Alleva et al., 2006; López-Pérez et al., 2009).

In relation to this, the lipid environment has been described being important and to determine the ability to enclose AQPs and therefore, to supply a tuned mechanism for adjusting and regulating both function and structure of membrane proteins (Engelman, 2005). Several studies showed that modifications in the

protein/lipid ratio of PM in *Brassica* species provided specific physical characteristic to the membranes establishing a salt tolerance range linked to the presence of membrane proteins (Chalbi et al., 2015). Besides, in an *in vitro* study, the aquaporin Z (AqpZ) was stabilized by lipids showing resistance to unfolding (Laganowsky et al., 2014), which support the discussion about the importance of the lipid environment in both protein function and stability. Thus, differences caused by changes in lipid environment and in protein/lipid ratio due to salinity stress could explain the higher stability in P_f values of NaCl PM vesicles.

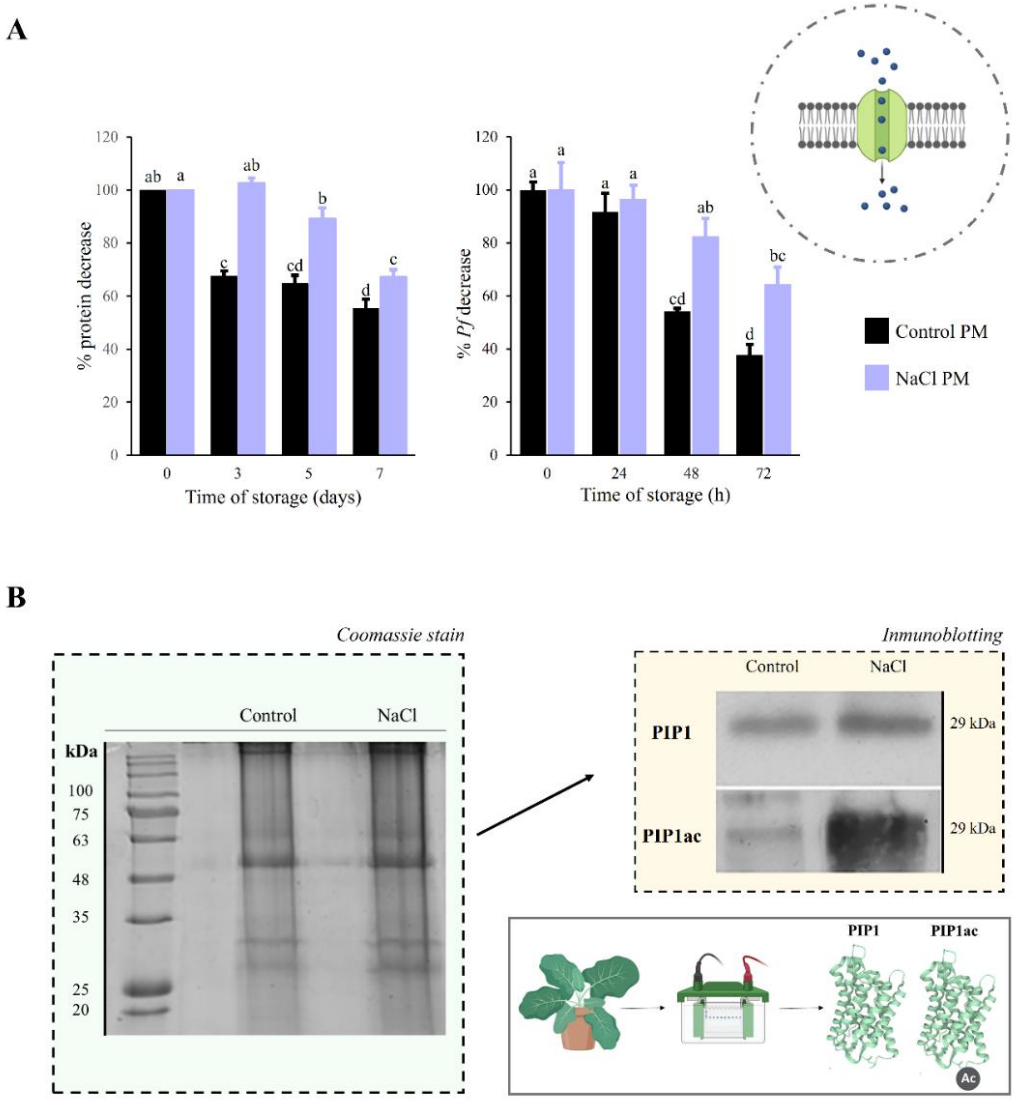

Figure 14. *Stability of membrane vesicles during storage. Protein degradation and decrease of the osmotic water permeability (P_f) over time (A). Coomassie stain and PIP1 and PIP1 acetylated (PIP1ac) homologues immunoblotting of plasma membrane (PM) proteins purified from broccoli roots both control and 100 mM NaCl (B). Data adapted from Martínez Ballesta et al., (2018).*

4.2.2. Post-translational modifications

On the other hand, post-translational modifications have been determined to be significant for defining the final location or adequate function of proteins. One of the most studied post-translational modification is the acetylation. N-terminal acetylation in soluble mammalian and yeast proteins has been extensively studied, although in plants and specifically in AQPs, this has been studied lesser extent. The biological role of this modification has been related with target of membrane, protein-protein interactions, alteration in proteostasis, increasing half-life of proteins, and resistance for degradation in cells (Polevoda and Sherman, 2000; Giglione et al., 2015). An increase in N-terminal acetylation in the methionine (Met) of PIP1 subfamily AQP in vesicles from salt-treated broccoli plants was shown (Figure 14B) and it was related to proteins stability by Martínez Ballesta et al., (2018).

4.2.3. Secondary structures

Results from Fourier-transform infrared spectroscopy (FTIR) analysis carried out by Martínez Ballesta et al., (2018) (Figure 15) showed both in control and NaCl membrane vesicles a reduction of β-turns and β-sheet secondary structures with the increase of temperature, which is due to a disruption of the structure of proteins provoke protein denaturation. Nevertheless, it could be argued that NaCl treatment would establish a more stable environment against temperature-induced denaturation, probably this could be due to changes in lipid composition of PM as a response to stress (Silva et al., 2007), protein expression or interaction between proteins and lipids. Regarding lipids, the vibrational stretching mode of the CH_2 group (3100-2800 cm^{-1}) provides information about the characteristics of the lipid membrane. In previous studies it was reported that salinity stress leads to an increase PM sterol content and membrane order, which provide more rigidity and better efficacy in the regulation of permeability to water (Silva et al., 2007; López-Pérez et al., 2009; Silva et al., 2011; Basyuni et al., 2012). The obtained results in this work regarding to CH_2 symmetric stretching band, showed NaCl membranes were more ordered than control membranes (Martínez Ballesta et al., 2018). The

displacement to a lower wavenumber in NaCl samples is related to a higher trans/gauche lipid ratio, which implies more viscosity and therefore producing more order membranes (Uematsu and Shimizu, 2021). This is in agreement with previous reports in which it was suggests that salinity causes modifications of lipid environment increasing the membrane rigidity (Liljenberg, 1992).

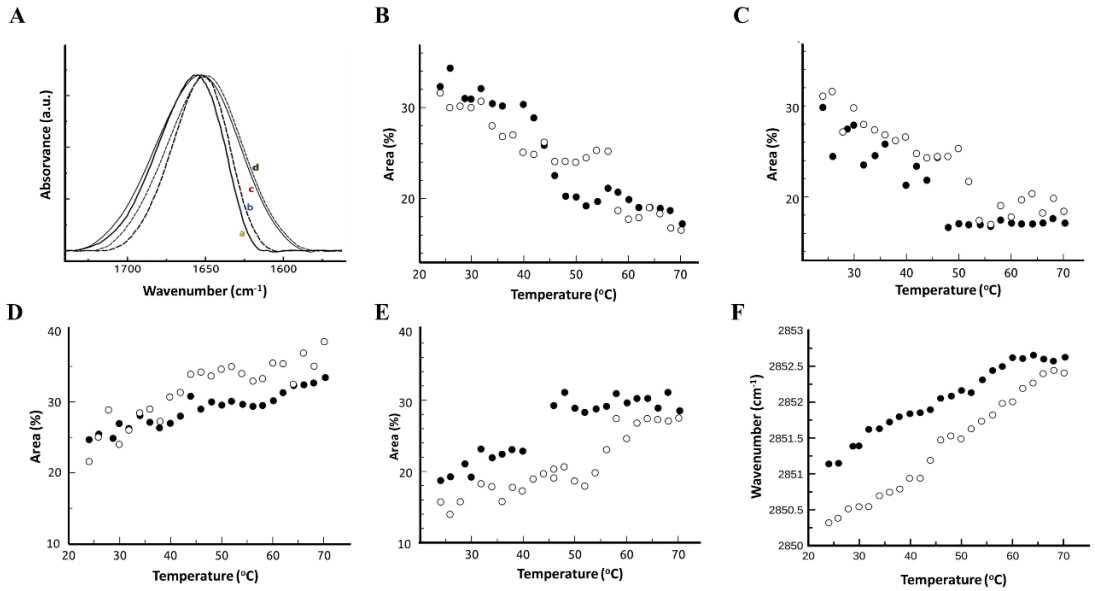

Figure 15. *Fourier-transform infrared spectroscopy (FTIR) of the Amide I band region of control plasma membrane (PM) at 24 ºC (a) and 70 ºC (b), and NaCl PM at 24 ºC (c) and 70 ºC (d) (A). β-sheet secondary structure (B), β-turns secondary structure (C), unordered and unfolded structures (D), α-helix secondary structure (E) and CH_2 symmetric stretching (F) of PM samples isolated from control plants (closed circles) and from plants treated with 100 mM NaCl (open circles). Data adapted from Martínez Ballesta et al., (2018).*

JUSTIFICATION AND OBJECTIVES

1. Justification

The use of encapsulation systems in different areas such as therapy, cosmetics, food, or agriculture has been widespread in recent years. These systems improve biotechnological applications, mainly when unstable compounds are used or when an increase of penetrability and mobility want to be achieved. Bioactive compounds from natural sources have very beneficial properties for human health. However, most of these compounds are unstable, and their physicochemical properties hinder their use in real applications and their industrial scale-up. The encapsulation of bioactive compounds in vesicular systems acting as protectors and vehicles for them was one way to overcome these drawbacks. On the one hand, these vesicular systems protect compounds from degradation and increase their stability. On the other hand, they can facilitate the penetration through biological barriers (skin, mucosal membranes, BBB, or enterocytes) to reach target sites and exert their function. There are numerous encapsulation systems with different properties, but they also have particular limitations, such as high cost or toxicity. It is therefore, necessary to develop new and more efficient encapsulation systems.

In addition, using agricultural by-products is a way to reduce waste and take advantage of resources, contributing to the development of a circular economy. In this sense, our research group as a result of previous studies, in which the effect of abiotic stresses at cell membrane level in broccoli plants was studied, raised the possibility that these membranes might have the potential to use as encapsulation systems. Thus, a new line of research emerged, which was opened with the patent entitled "*Method for obtaining plasma membrane vesicles extracted from plants enriched in membrane transport proteins, and uses thereof*" (Patent EP2716280).

As a consequence of the above, and in order to advance in the emerged research line about membrane vesicles from plants, this PhD thesis was raised, and the following main and specific objectives were set.

2. Objectives

The **main objective** of this PhD thesis is to advance the development of membrane vesicles from *Brassica oleracea* L. var. *italica* (broccoli), specifically from by-products, for use them as nanocarrier and drugs in biotechnological applications related to cosmetics or pharmaceuticals application.

In order to reach the general aim, three **specific objectives** were proposed matching with the three chapters in which the thesis has been structured (Figure 16).

1. To elucidate the potential of membrane vesicles extracted from broccoli to penetrate the inner layers of the skin and evaluate their ability to release and deliver encapsulated compounds to skin cells. As well as to determine the stability of the vesicles in a real cosmetic formulation (***Chapter I***).

2. To elucidate the capacity of membrane vesicles from broccoli to encapsulate the bioactive compound SFN and their application in a skin cancer cell line (***Chapter II***).

3. To determine the anti-inflammatory potential of broccoli membrane vesicles and encapsulation with SFN in a human-macrophage-like *in vitro* cell model under both normal and inflammatory conditions (***Chapter III***).

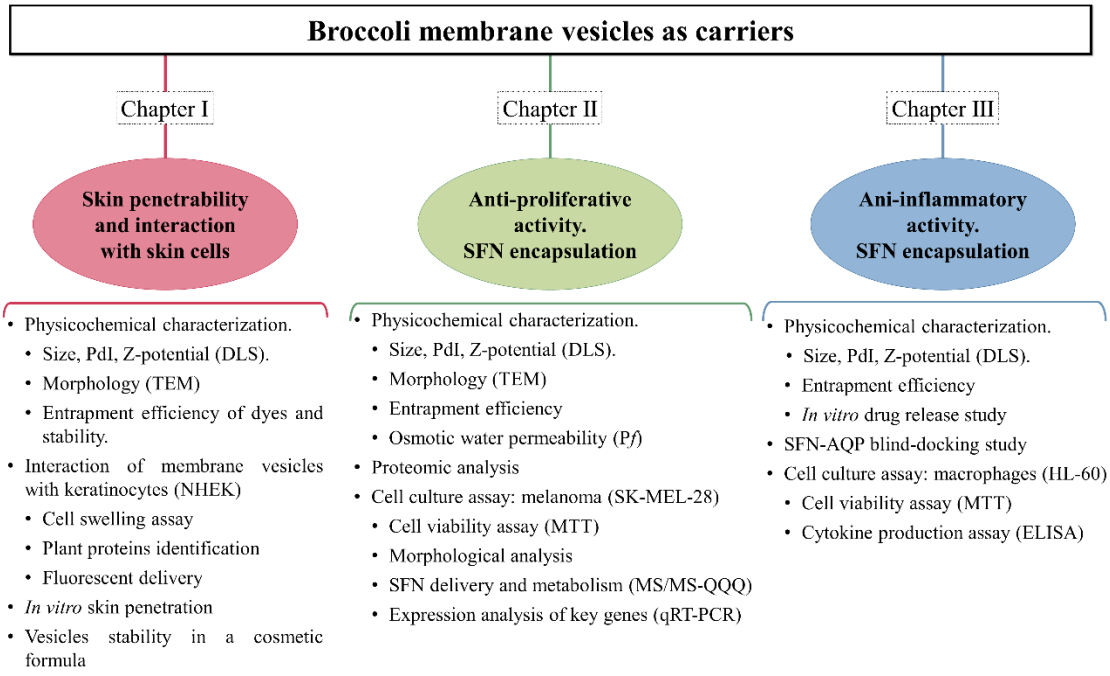

Figure 16. *Scheme of the thesis structure. The main aspects studied are shown for each chapter with "Broccoli membrane vesicles as carriers" as the central axis of this thesis.*

RESULTS

Chapter I

Plant plasma membrane vesicles interaction with keratinocytes reveals their potential as carriers

Chapter I: Plant plasma membrane vesicles interaction with keratinocytes reveals their potential as carriers

Lucía Yepes-Molina, Maria Carmen Martínez-Ballesta and Micaela Carvajal.

Journal of Advanced Research, 23 February 2021 | doi.org/10.1016/j.jare.2020.02.004

During the last few years, membrane vesicles (as exovesicles) have emerged as potential nanocarriers for therapeutic applications. They are receiving attention due to their proteo-lipid nature, size, biocompatibility, and biodegradability. In this work, we investigated the potential use of isolated root plasma membrane vesicles from broccoli plants as nanocarriers. For that, the entrapment efficiency and integrity of the vesicles were determined. Also, the delivery of keratinocytes and penetrability through skin were studied. The results show that the broccoli vesicles had high stability, in relation to their proteins, and high entrapment efficiency. Also, the interaction between the vesicles and keratinocytes was proven by the delivery of an encapsulated fluorescent product into cells and by the detection of plant proteins in the keratinocyte plasma membrane, showing the interactions between the membranes of two species of distinct biological kingdoms. Therefore, these results, together with the capacity of brassica vesicles to cross the skin layers, detected by fluorescent penetration, enable us to propose a type of nanocarrier obtained from natural plant membranes for use in transdermal delivery.

Chapter II

Nanoencapsulation of sulforaphane in broccoli membrane vesicles and their in vitro antiproliferative activity

Chapter II: Nanoencapsulation of sulforaphane in broccoli membrane vesicles and their *in vitro* antiproliferative activity

Lucía Yepes-Molina and Micaela Carvajal.

Pharmaceutical biology, 7 October 2021 | doi.org/10.1080/13880209.2021.1992450

The development of nanocarriers of plant origin, such as plant cell membranes, has recently been investigated. Also, plant bioactive compounds as sulforaphane (SFN) from broccoli have recognized antioxidant or anticancer properties. To investigate the capacity of membrane vesicles from broccoli (BM-vesicles) to encapsulate SFN and their application in the cancer cell line. Physicochemical analysis was carried out to characterize BM-vesicles through different approaches: dynamic light scattering, transmission electron microscopy, stopped-flow analysis, and proteomic analysis. They were applied at different concentrations (BM-vesicles at 0.04–0.00315% of protein and SFN at 5, 25, and 100 mM) in SK-MEL-28 cells during 24 h for studying cytotoxicity and gene expression. The entrapment efficiency was 41%. The anticancer activity tested in cells showed a decrease in proliferation when SFN in BM-vesicles was utilized. Expression patterns when SFN was applied in an encapsulated form showed a reduction of cancer markers and an increase of AQP3. Also, the metabolism of SFN occurred inside of cells, and higher SFN penetrated when it was encapsulated. The results showed that encapsulated SFN was better absorbed by melanoma cells providing metabolism products and a reduction of cancer molecular markers. Also AQP3 was pointed to as an important marker since it appeared to play a key role in homeostasis due to the importance of water transport in biological processes. As conclusion, these results indicate that SFN and SFN encapsulated in BM-vesicles have a high activity for the inhibition of melanocyte development. Therefore, BM-vesicles could serve as nanocarriers for drugs.

Chapter III

Membrane vesicles for nanoencapsulated sulforaphane increased their anti-inflammatory role on an in vitro macrophage model

Chapter III: Membrane vesicles for nanoencapsulated sulforaphane increased their anti-inflammatory role on an *in vitro* human macrophage model

Lucía Yepes-Molina, María Isabel Pérez-Jiménez, María Martínez-Esparza, José A. Teruel, Antonio J. Ruiz-Alcaraz, Pilar García-Peñarrubia and Micaela Carvajal.

Int. Journal of Molecular Sciences, 9 February 2022 | doi.org/10.3390/ijms23041940

At present, there is a growing interest in finding new non-toxic anti-inflammatory drugs to treat inflammation, which is a key pathology in the development of several diseases with considerable mortality. Sulforaphane (SFN), a bioactive compound derived from *Brassica* plants, was shown to be promising due to its anti-inflammatory properties and great potential, though its actual clinical use is limited due to its poor stability and bioavailability. In this sense, the use of nanocarriers could solve stability-related problems. In the current study, sulforaphane loaded into membrane vesicles derived from broccoli plants was studied to determine the anti-inflammatory potential in a human-macrophage-like *in vitro* cell model under both normal and inflammatory conditions. On the one hand, the release of SFN from membrane vesicles was *in vitro* modelled, and two release phases were stabilized, one faster and the other slower due to the interaction between SFN and membrane proteins, such as AQPs. Furthermore, the anti-inflammatory action of sulforaphane-loaded membrane vesicles was demonstrated, as a decrease in interleukins crucial for the development of inflammation, such as TNF-α, IL-1β and IL-6, was observed. Furthermore, these results also showed that membrane vesicles by themselves had anti-inflammatory properties, opening the possibility of new lines of research to study these vesicles, not only as carriers but also as active compounds.

GENERAL DISCUSSION

General Discussion

This section aims to provide a general overview of the research undertaken in this PhD Thesis, integrating all the results obtained in the different chapters and discussing the prospects of this area of work as well as the possible applications derived from this research. Therefore, this PhD thesis focused on developing new encapsulation systems based on broccoli plants by-products and testing the potential of the obtained membrane vesicles in cosmetic and therapy applications through *in vitro* studies.

1. Profitability of using plants as source of drug delivery systems

Broccoli, together with other Brassicas, is a crop widely spread in the Region of Murcia, and a great amount of by-products are derived from their agricultural exploitation. Finding an application for them is an objective to be pursued since this would favour economic activity on the one hand and the environment on the other (Domínguez-Perles et al., 2010). In this way, the available resources and sustainable materials such as crop by-products offers a high profitability. These materials can be harvested on a large scale and they can come from different sources. In the case of crops such as broccoli, by-products are mainly inedible parts such as roots and leaves (Domínguez-Perles et al., 2010). Both materials are suitable for obtaining membrane vesicles. The research performed during this PhD thesis began with vesicles obtained from broccoli roots, but they would be problematic to get from a crop and consequently it would involve extra work, and from an industrial and commercial point of view, this would increase the final cost of vesicles production and limit the applications of the same due to the decrease in the economic profit margin. For this reason, another part of the broccoli plant was tested and subsequent trials of this thesis were conducted with membrane vesicles from broccoli leaves, which are a large part by weight of the by-products of broccoli crops, and unlike roots, leaves are easy to harvest and neither are an edible part of broccoli plants. In addition, from non-edible parts, it is also possible to

obtain vesicles from edible parts. Recently, several studies have been developed with vesicles obtained from edible parts of plants such as ginger, grape, lemon, grapefruit, carrot, and broccoli and cauliflower inflorescence (Kameli et al., 2021; Garcia-Ibañez et al., 2022; Wang et al., 2022). Although from these edible parts good yield and vesicles with suitable properties to use for example in therapy applications are obtained, it can be counterproductive in the sense that materials are intended for human consumption. This problem would therefore not be present whether non-edible by-products are used, as in our case. In addition, it is important to highlight that this thesis addresses objectives framed within the European bioeconomy strategy, which raise five goals: to ensure nutrition security, to manage natural resources sustainably, to reduce dependence on non-renewable resources, to adapt and limit climate change, and to create jobs strengthening European competitiveness (Italian Government, 2019).

1.1. Advantages of plant-derived vesicles

The use of vesicular systems to encapsulate and protect compounds of interest in several fields such as cosmetic, therapy, or food has been going on for many years. In fact, liposomes were discovered in 1960, and in 1990 FDA approved a drug based on this technology (Daraee et al., 2016). Since then, many breakthroughs have been achieved in the development of improved drug delivery systems (DDS), since liposomes showed from the beginning a relatively high mechanical instability, as they show degradation and precipitation in short time (Allahou et al., 2021). In this respect, searching new DDS is a potential research area (Zhong et al., 2018), and specifically the search of DDS from natural sources has been recently increased. In the last few years, several works carried out by our group and others have drawn attention to the potential could have mammals and plant-derived membrane vesicles as candidates for therapeutic, cosmeceuticals, and drugs delivery applications in general (Ali et al., 2022). Due to their therapeutic activity, safety, scalable productivity, and stable structure associated with the presence of proteins together with lipids and the strong unions among

them (Martínez Ballesta et al., 2016; Martínez Ballesta et al., 2018; Wang et al., 2022).

Plant-derived vesicles from different species have been highlighted in several studies not only by their low cytotoxicity and biodegradability compared to other DDS (Nemati et al., 2022), but also by their biocompatibility and interaction with human cells (Ju et al., 2013; Wang et al., 2013; Garaeva et al., 2021), which allow the development of multitude of applications in therapy treating diseases such as inflammation related disorders or cancer (Wang et al., 2022). Furthermore, plant-derived membrane vesicles, unlike mammalian vesicles obtained from cell culture supernatants or fluids such as plasma, saliva, urine or milk, present unique advantages such as being undetected by the immune system, plants are free of zoonotic or human pathogens and are innocuous (Dad et al., 2021).

Cytotoxicity of DDS is one of the most important characteristics to study in the initial stages of drug or cosmetic development. Liposomes, the most similar DDS to plant membrane vesicles and most widely studied, are defined by their low toxicity, since they are generally composed of phospholipids. Previously, it has been established that cytotoxicity is not only determined by the application dose, but also the composition, size, charge, shape, and solubility (Roursgaard et al., 2016). Toxicity induced by liposomes was determined at high doses of 0.1-1 mg mL^{-1} for example in HepG2 cell culture (hepatic cell line) (Roursgaard et al., 2016). Although in the same study, micelles displayed lower toxicity than liposomes, maybe because of PEGylation avoids a strong recognition by immune cells. This fact is interesting since it supports subsequent studies where the surface of the vesicles is modified or natural vesicles with specific composition are sought that make them less recognizable by the immune system and therefore less toxic (Roursgaard et al., 2016).

Regarding plant membrane vesicles, numerous studies are recently being carried out in which cytotoxicity is tested in different cell lines (Kim et al., 2020). The low toxicity in cell cultures has been checked similar to liposomes, and in addition it has also been determined that this toxicity will depend on the target

tissue, for example, whether cells are normal or carcinogenic (Kim et al., 2020), which is in accordance with results obtained in this thesis. Thus, *in vitro* toxicity assays were carried out in two cellular models: human skin cancer cell model (melanoma) (**chapter II**) and human macrophage-like cell model (studied in both homeostasis and inflammatory conditions) (**chapter III**). At similar protein concentrations, the membrane vesicles showed cytotoxicity of around 50% in cancer cells, however, they did not show a high toxicity to same concentration in macrophages. This relative high cytotoxicity in carcinogenic cells could be interesting in cancer studies, since it is important a specific and targeted toxicity for malignant cells. Hence, based on these results, different possibilities open up to deepen the use of this type of membrane vesicles to treat cancer. Viewed in this way, it is essential that plant membrane vesicles are innocuous in normal cells and other previous works support it. Membrane vesicles isolated from other brassica plants such as cauliflower and cabbage did not show a significant decrease in cell survival rate when they were applied in HepG2 hepatocytes, HaCaT keratinocytes, HDF fibroblast, and RAW264.7 macrophages (Yepes-Molina et al., 2021; You et al., 2021; Garcia-Ibañez et al., 2022).

As regard biocompatibility between plant and human cells (Mu et al., 2014), interaction among different kingdoms is bringing out and membrane vesicles could be essential to study it. For example, plant-fungal interactions have been detected through EVs but also between plants and mammals through feeding, which has been the starting point to deepen this aspect, and to study the interaction between membrane vesicles derived from plants and mammalian targets (Pérez-Bermúdez et al., 2017; Urzì et al., 2022). Thus, in this thesis an interaction between broccoli membrane vesicles and human cells, specifically keratinocytes, has been established. It was shown that AQPs from broccoli membrane vesicles remained in keratinocytes membranes after application, revealing also a contribution of these plant proteins in the cell swelling after the application of water to keratinocytes cell culture (**chapter I**). Results from this research support the emerging interest in determine the details about the cross-kingdom interaction. In addition to a physical interaction at the level of both membranes, different works have already

established that the interaction between plant material such as membrane vesicles and mammalian targets can trigger genetic regulation mediated by miRNAs contained in plant vesicles (Urzì et al., 2022). This fact is relevant since it could be the basis for developing novel strategies in human therapies based on natural resources. So, following this line of research, it would be interesting to delve into what refers to the miRNAs containing in the membrane vesicles of broccoli, which could be responsible, along with other secondary metabolites, for the self-bioactivity of these vesicles, which is discussed later.

1.2. Applications of plant membrane vesicles

Plant membrane vesicles, like other NPs, have been proposed to be used as DDS to encapsulate different types of compounds. Thus, encapsulation was mainly proposed to cope with two general drawbacks: 1) the difficulty of compounds or drugs to cross biological barriers and to reach the final target sites (Yang et al., 2017), and 2) the *in vitro* and *in vivo* instability of bioactive compounds (Pachuau et al., 2021).

Skin is the first biological barrier of organism, as it is the most external organ exposed to the environment. Stratum corneum with 10-20 μm-thick is the outermost skin layer, and is composed by packed and keratinized dead cells, therefore stratum corneum represents the primary barrier to drug permeation (Elias, 2005). Some drugs can pass through the skin without enhancement mechanisms, but most will have difficulty doing so. It has been described that drugs with low molecular weight and *log P* (logarithm of the octanol-water partition coefficient of a molecule) between 0 and 5 must be able to cross this barrier (Kanfer and Shargel, 2020). Even so, the list of drugs capable of crossing the stratum corneum has been increased by using enhancers. One of the enhancers most popular is the encapsulation in vesicular structures or NPs, which has been used to improve the penetration of drugs into target tissues.

Thus, in this PhD thesis, we have proposed using broccoli membrane vesicles to encapsulate drugs and improve permeability across biological barriers such as the stratum corneum. Membrane vesicles were labelled with a fluorescent

compound (unable to penetrate the inner layers of the skin), and it was shown that broccoli membrane vesicles cross the stratum corneum and reach the inner layers of the skin (**chapter I**). Permeation seems to happen through transcellular route along hair follicles, which has been considered the main path for NPs with sizes between 70 and 500 nm (Yang et al., 2017; Carter et al., 2019), since the size of our vesicles is about 350 nm. Besides, it has been established in studies carried out with liposomes that size of vesicles has a significant influence on delivery of compound into the skin and a diameter higher of 600 nm are not able to entry into inner skin layer, but nanovesicles with a size around 300 nm are able to deliver their encapsulated compounds into deep skin layers (Danaei et al., 2018).

As mentioned above, in addition to facilitating drugs to cross biological barriers and increasing cell/tissue interactions, encapsulation of compounds is also proposed to improve the physicochemical properties of them, such as increasing drug stability and reducing adverse effects, leading to obtain more effective and less toxic treatments (Frank et al., 2015). This is an important point, since many bioactive compounds are generally unstable both *in vitro* and *in vivo* conditions, and they are susceptible to oxidative degradation (Shishir et al., 2018). Therefore, once broccoli membrane vesicles have been reported as suitable DDS in skin therapies, the following assays were raised to encapsulate SFN in vesicles and apply them in cell cultures in order to explore specific applications in skin pathologies of broccoli membrane vesicles. SFN, the main ITC of broccoli plants with multiple beneficial effects as anti-oxidant, anti-inflammatory and anti-carcinogenic activity (Dinkova-Kostova and Kostov, 2012), has been the focus of many clinical preliminary studies due to its great potential as health-promoting compound (Quirante-Moya et al., 2020). However, its industrial scale up has limitations since SFN is a very unstable compound (Van Eylen et al., 2007; Zambrano et al., 2019). In order to improve SFN stability, encapsulation has been proposed as a promising approach to increase the *in vivo* functionality and bioavailability of SFN, since this technique protect bioactive compounds from degradation associated to external conditions (Zambrano et al., 2019; Yuanfeng et al., 2021).

On the basis of the positive results obtained with other DDS, SFN was encapsulated in broccoli membrane vesicles and efficacy of this encapsulation was determined by applied studies to check the anti-carcinogenic (**chapter II**) and anti-inflammatory effects (**chapter III**) of encapsulated SFN compared to free SFN. These preliminary studies in cell culture mainly revealed that encapsulation of SFN in plant membrane vesicles is feasible, as the properties of SFN are maintained in the encapsulation systems. Therefore, further studies could be conducted to advance and achieve future clinical applications with SFN or similar bioactive compounds encapsulated in plant-derived vesicles.

Although in two different cell models (melanoma and macrophages), the studies can be related to each other since both were investigated under two premises: 1) the anti-inflammatory activity of SFN by inhibiting the NF-κB signaling pathway, which activate inflammatory mediators such as IL-6, IL-1β, and TNF-α (Dinkova-Kostova and Kostov, 2012; Ruhee and Suzuki, 2020), and 2) the inflammatory component of many skin diseases, including cancer such as melanoma, the most dangerous skin cancer, and another based on a chronic inflammation (psoriasis, atopic dermatitis, or rosacea) (Lee et al., 2021), in which macrophages have a critical role in initiation, maintenance, and resolution of the inflammatory process (Fujiwara and Kobayashi, 2005). Skin is home to abundant populations of cells including macrophages (Ho and Kupper, 2019), which related to cancer progression are principal components by facilitating invasion, immunosuppression, metastasis, and angiogenesis (Poh and Ernst, 2018). Specifically, macrophages are a key element in the melanomagenesis in skin melanoma (Pieniazek et al., 2018).

In our results, we observed that the changes caused by SFN in the cell markers studied (p53, BAX, AQP3, TNF-α, IL-6, and IL-1β) (**chapter II** and **III**) did not show important differences between encapsulated and free SFN. However, the fact that cell assays are generally performed in the short term, specifically these at 24 h points to the encapsulation advantages would be determine in the long-term experiments (more than 48 h) (Zanotto-Filho et al., 2013). Therefore in the

following investigation stage scaling from *in vitro* cell culture assays and extending studies to animal experiments should be planned to test encapsulation system in the long-term (Frank et al., 2015).

We tested our vesicles to encapsulate SFN, and in these preliminary assays, interesting results were obtained. As well as other works from our group support and extend these results showing membrane vesicles derived from cauliflower are suitable for encapsulating and stabilizing plant extracts, for example, from pomegranate, Bimi®, or red cabbage (Garcia-Ibañez et al., 2021; Yepes-Molina et al., 2021; Garcia-Ibañez et al., 2022). In addition, other works unrelated to our investigations have shown that different vesicles or NPs derived from plants are suitable for encapsulating various types of compounds, both hydrophobic and hydrophilic, and facilitating their release in a targeted manner to the target site. For that, it is necessary to cross biological barriers as skin and encapsulation also improving this fact. Among encapsulated compounds there are chemotherapeutic agents, proteins, and genetic material such as short interfering RNA or DNA expression vectors (Wang et al., 2013).

2. Self-bioactivity of plant membrane vesicles

Furthermore, derived from the studies carried out in this thesis with SFN encapsulations, interesting results were obtained about the effect of broccoli membrane vesicles by themselves. For example, the cytotoxic effects in cancer cells as resumed above or the modulation of BAX, AQP3, TNF-α, IL-1β, and IL-6 levels. Plant-derived vesicles are mainly composed of lipids and proteins. But they also have associated biomolecules in their lumen and surface, and some of them have bioactivity such as ITCs and phenolic compounds, or nucleic acids as miRNA, which are able to modulate gene expression in human target cells (Urzì et al., 2022). Thus, plant-derived vesicles can be used both as DDS and drugs. Therapeutic effects of plants have been studied in depth for many years, and some of these effects remain in vesicles derived from different plant tissues (Nemati et al., 2022). The results obtained in this thesis related to the bioactivity of broccoli membrane vesicles are supported by other studies done with NPs derived from

edible plants (grapes, ginger, grapefruit, citrus, or carrot), in which anti-cancerous and anti-inflammatory properties were revealed (Ju et al., 2013; Wang et al., 2014; Raimondo et al., 2015). Hence, anti-inflammatory activity of plant membrane vesicles could be attributed to ITC, phenolic compounds, some lipids and miRNAs (Zhang et al., 2022). For example, lipids of vesicles from broccoli induced CD11and inhibit gut inflammation (Deng et al., 2017), miRNAs (miR159a and miR156c) containing in nuts derived vesicles showed anti-inflammatory effects by dampen TNF-α signalling (Aquilano et al., 2019), and vesicles from grapefruit, ginger and carrot trigger activation of Nrf2 in macrophages (Mu et al., 2014).

Additionally, the possibility of modulating vesicles characteristics is an advantage when using them as a drug. Thus, depending on species, tissues, and plant growth conditions, for example, if plants grown under stresses, membrane vesicles with different compositions in lipids, proteins, and associated bioactive compounds could be obtained (Chalbi et al., 2015; Yepes-Molina et al., 2020). On the one hand, lipid composition has a direct effect on the biological function of plant membrane vesicles, and a specific lipid profile provides to vesicles detailed functional advantages (Liu et al., 2020), being key in mitogenesis, fission processes, modulation of the intestinal microbiota, and membrane fusion. For example, the presence of phosphatidic acid (PA) and phosphatidylcholine (PC) in vesicles derived from ginger was significant in migration from the intestine and uptake by intestinal bacteria (Chen et al., 2019). Although advances have been made regarding the lipid characterization of plant membrane vesicles, a better understanding of the structure and composition of the entire variety of lipids is required, since lipids have been analysed as a whole. However, the individual role of each type of lipid has not yet been elucidated. Besides, while in the study of plant membrane vesicles mainly phospholipids are analysed, making advanced in fatty acids research could be interesting, so these are key in parameters as membrane rigidity or fluidity, which might be affect to encapsulation and delivery of compounds, and fusion of vesicles with target cells (Bompard et al., 2020). On the other hand, as in the proteomic characterisation of vesicles, a similar scenario occurs with lipids, groups of proteins predominantly found in plant-derived

vesicles have been identified as proteins involved in metabolic signaling, cellular transport, or secretory pathways, highlighting annexins, actins, AQPs, clathrins, or heat shock proteins (Nemati et al., 2022). Focusing on AQPs, a correlation between these transmembrane proteins and vesicle stability has been established (Martínez Ballesta et al., 2018), although more investigation is required to advance the mechanisms of action. So, integrating both proteomic and lipidomic complete analysis, may be key for developing therapeutic strategies based on this type of vesicles.

The results obtained here, together with those in the literature on therapeutic and cosmetic applications of plant-derived membrane vesicles, such as anti-inflammatory and anticancer potential, validate their deserved attention in the fields of biomedicine and cosmeceuticals. Although the research on plant-derived membrane vesicles from an applied point of view is recent, some clinical studies are already underway. For example, there are studies with vesicles derived from ginger, aloe, or grapes to treat colon cancer, insulin-related conditions, chronic inflammation, or oral mucositis (Nemati et al., 2022). Therefore, investigation carried out in this thesis is at a point with multiple possibilities, and several work lines are open after this PhD thesis.

3. Physicochemical properties of plant membrane vesicles will determine their potential use

Different factors must be considered to use membrane vesicles as DDS since physicochemical properties have got a high impact on the systemic activities and molecular biology of DDS. The most significant parameters are size, polydispersity, and Z-potential (surface charge) (Raval et al., 2018). It is possible to modulate the size of the membrane vesicles obtained from plants, mainly depending on the purified membrane fraction. Open the possibility that vesicles with different properties were obtained from the same source. Sizes from 200 to 500 nm were determined depending on whether vesicles were from MF, PM, or DRMs (Martínez Ballesta et al., 2018; Yepes-Molina et al., 2020) (**chapter I** and **II**). Although, adjusting their sizes by filtration techniques is possible once these

fractions are purified (**chapter III**). Thus, in assays carried out on macrophages, MF vesicles with an average size of 200 nm were used. Regarding, Z-potential, membrane vesicles had a negative charge of around -30 mV. A surface with a negative charge is appropriate for topical DDS, which is in line with mentioned above regarding the use of vesicles in skin therapies by transdermal drug delivery. Different studies have shown negatively charge liposomes are more efficient in drug penetration through skin compared to positively charge and neutral charge liposomes (Sinico et al., 2005; Gillet et al., 2011).

Z-potential is also a value that offers information about dispersion stability. Values higher than +25 mV and lower than -25 mV are a reference for stability, as repulsive forces between particles avoid aggregation (Vallar et al., 1999). The stability and integrity of vesicles should be determined from their production to their application to determine the shelf-life of the final products containing DDS. Previously, broccoli membrane vesicle integrity and functionality were determined through water transport capacity, mainly carried out by the AQPs, whose presence in lipid membranes was related to *in vitro* vesicles stability (Martínez Ballesta et al., 2018). As mentioned above, vesicles that include proteins tend to be more stable because the covalent bonds between lipids and proteins give them stability (Seneviratne et al., 2018). Post-translational modifications of proteins such as acetylation regulate protein stability as well as interaction with other proteins and macromolecules (Mittal and Saluja, 2015; Zecha et al., 2022). This could be related to membrane vesicles stability, so an increase in acetylation of AQPs was related to higher stability of vesicles over time (Martínez Ballesta et al., 2018). In addition, proteins in natural membrane vesicles are also anchor points for encapsulated substances. For example, in a previous study carried out by our group, AQPs in PM vesicles were shown as potential stabilizers of glucoraphanin (GRA), and specific interactions between the alkane chain of GRA and two protein residues were shown (Martínez Ballesta et al., 2016). In a similar way, interactions between residues of conserved motif NPA of AQPs and the bioactive compound SFN were shown in this thesis (**chapter III**). Besides, as an approximation to real application and to estimate the shelf-life, membrane vesicles were added to a cosmetic

formula. After one year, a high percentage of integral and functional vesicles remained in the cream (**chapter I**).

The physicochemical characteristics of the vesicles, such as their size, will determine the entry pathway to the target cells/tissues and establish the most appropriate administration route for cosmetic or therapeutic application. In this respect, advanced research about the interaction between broccoli membrane vesicles and human cells and the delivery to the target of the encapsulated compound in vesicles was not carried out in this thesis. Although the fusion between plant and human membranes in the study carried out with human keratinocytes is proposed (**chapter I**). At this point, we must establish future objectives to confirm our hypothesis.

In addition to membrane fusion, there are other vesicle internalization and delivery types, such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis (Mulcahy et al., 2014). In a study with membrane vesicles from *Aloe vera*, the different routes of vesicle internalization were quantitatively analysed. The results showed that vesicles enter cells by three pathways: membrane fusion and clathrin- and caveolae-mediated endocytosis (Kim et al., 2021). Based on these results, we could hypothesize a similar behaviour of our broccoli membrane vesicles since both are derived from plants. Although, vesicle size and cellular type of target must be considered, and the percentage of each route could change. In the case of macrophages, phagocytosis (another type of endocytosis) could be the majority pathway. Phagocytosis consists of the internalization of opsonized particles, which is carried out by specialized cells such as macrophages (Luu and Maurel, 2013). In addition to the most studied mechanisms, others are beginning to be considered.

For example, endocytosis in which lipid rafts are involved (Mulcahy et al., 2014), the interaction with the proteins located in the raft domains will be essential, where a specific composition of lipids and proteins appears. Lipid rafts contribute to viral particle uptake by cells through glycoprotein binding and adjusting the properties of the membrane (Teissier and Pécheur, 2007; Mulcahy et al., 2014).

Thus, knowing the receptors in these domains can lead to the design of membrane vesicles to be uptaken by specific cells. Generally, in the bibliography, there is no agreement regarding the mechanisms of internalization of vesicles or the encapsulated compounds by the target cells. Based on all current results, the most probable way is that different mechanisms are triggered simultaneously. However, one can predominate over another depending on the characteristics of vesicles (size, heterogeneity, or lipid-protein composition) and the nature of target cells.

Finally, the last question to be addressed is the possible routes of administration of membrane vesicles as a drug in therapy or cosmetic application. For the administration of liposomes, systems more similar to our membrane vesicles and already approved in some applications have been tested several routes: parenteral, transdermal, oral/nasal inhalation, and ocular (Maja et al., 2020). Until now, the parenteral route is the prevalent one for clinically approved treatments (Solomon et al., 2017). Although, the other options have also been explored. The transdermal route for drug delivery has the advantage over other routes of administration in that the gastrointestinal tract is avoided (Gillet et al., 2011). Encapsulated drugs are applied to the skin locally to penetrate the skin and exert a systemic effect. This route of administration has been used to apply treatments against inflammation, which is one of the focuses of this thesis, in diseases such as rheumatoid arthritis and psoriasis (Xi et al., 2013; Chaudhary et al., 2014). As stated above, our vesicular system based on broccoli membrane vesicles would be suitable to apply through a transdermal route since they were characterized for their capacity to cross the stratum corneum and deliver the encapsulated compounds into the inner skin layers (**chapter I**).

Regarding the oral route of drug administration, the degradation of compounds in the gastrointestinal tract should be a problem. Recently several studies have shown that this route could also be an effective and safe way to administrate encapsulated compounds. Plant-derived membrane vesicles resist the acidic environment in the stomach and are absorbed in the intestinal tract (Wang et al., 2022). Recently it has been reported that bioactive compounds derived from plants

and encapsulated in cauliflower membrane vesicles could exert their function after gastrointestinal digestion (Garcia-Ibañez et al., 2021; Garcia-Ibañez et al., 2022).

4. Concluding remarks

In summary, plant-derived membrane vesicles can be obtained in large amounts and are cost-effective way; beside, they are biocompatible with mammalian cells and therefore safe as effective vesicles, which could act as both therapeutic drugs and delivery systems. At the beginning of this thesis, little was known about the possibilities of plant-derived membrane vesicles in biotechnological applications, and only a few works had been published in this area. Nevertheless, in the last five years, great advances have been made in this sense, and many studies have been carried out with vesicles obtained from various plant sources such as carrot, grape, grapefruit, ginger, nut, lemon, etc. So, the great potential to use these vesicles in therapy, cosmetics, or food has been confirmed.

Thus, membrane vesicles obtained from broccoli by-products in this thesis will have great potential for drug delivery or as drug by themself in the treatment of diseases and cosmetic applications in the near future. Broccoli membrane vesicles could be incorporated as a complementary or main component of cosmetic formulations or drugs for topical application in order to treat disorders related to inflammation and oxidative stress, such as diseases or skin disorders (psoriasis, acne, melanoma, etc.), since they are stable in a real cream formula, and are capable of crossing the stratum corneum. In addition, the ability of these vesicles to encapsulate compounds with high interest due to their bioactivity, such as SFN, and to release their content in both normal and tumour cells has been shown. On the other hand, in this thesis it has been revealed the self-activity of broccoli derived membrane vesicles to modulate different inflammatory related mediators both in human melanoma cells and human macrophages. Although as these are vesicles purified from natural sources and composed of a complex mixture of lipids, proteins, and others, it is challenging to establish specifically what their bioactivities are due to. *In vitro* stability of membrane vesicles was related to the

presence of transmembrane proteins such as AQPs, which have also been shown to act in the stabilisation of encapsulated compounds, and thus to play a role in the controlled release of compounds. In addition, proteins with an antioxidant profile and ITCs have been detected in broccoli vesicles. Following this line of work, it would be interesting to consider in the future carrying out precise characterisations of the systems in terms of proteins, lipids, nucleic acids, and other associated bioactive compounds in order to elucidate specific mechanisms of action and to be able to design precise therapeutic strategies.

CONCLUSIONS

Conclusions

From the results obtained in the different chapters of this PhD thesis and regarding the specific objectives established, the following conclusion can be drawn:

Objective 1: To elucidate the potential of membrane vesicles extracted from broccoli to penetrate the inner layers of the skin and evaluate their ability to release and deliver compounds encapsulated in them to skin cells. As well as to determine the stability of the vesicles in a real cosmetic formulation (Chapter I).

I. Broccoli root plasma membrane vesicles showed high entrapment efficiency using two dyes as model compounds, and showed stability under *in vitro* conditions and in a real cosmetic formulation for one year, maintaining enough amount of protein for adequate functionality.

II. Broccoli root plasma membrane vesicles encapsulating a fluorescent dye, released it to both cell culture human keratinocytes and in the inner layers of a pig skin disc. Thus, the vesicles were able to cross the PM of keratinocyte and the impermeable surface barrier of the skin (stratum corneum).

III. The application of broccoli root plasma membrane vesicles in cell cultures (a human keratinocyte cell line) allowed to show interactions between plant and human cell membranes, highlighting the role of aquaporins in this contact. Crossover interactions between both kingdoms leading open many lines of investigation and numerous potential applications.

Objective 2: to investigate the capacity of membrane vesicles from broccoli to encapsulate the bioactive compound SFN and their application in a skin cancer cell line (Chapter II).

IV. Microsomal fraction vesicles from broccoli leaves are very efficient in encapsulating the bioactive compound sulforaphane (SFN), being the most important isothiocyanate (ITC) in broccoli. After encapsulation the

properties of vesicles did not change, maintaining size, polydispersity, Z-potential, and osmotic water permeability. Furthermore, SFN did not alter its bioactivity *in vitro* assays upon encapsulation.

V. Analysis of the composition of vesicles obtained from broccoli leaves microsomal fraction revealed that they contain bioactive compounds such as ITCs and indoles like SFN, erucin, iberin or indole-3-carbinol (I3C), and proteins associated with antioxidant activity, important in self-activity of vesicles, and proteins related to binding activity, which could be key in the fusion with target cells.

VI. SFN encapsulated in broccoli membrane vesicles showed high inhibition activity of melanocyte development when applied for 24 h in cell culture and triggered an increase of the p53 antioncogene, which gives rise to a tumour suppressor protein.

VII. SFN in both free and encapsulated form, and broccoli membrane vesicles, up-regulated AQP3 (aquaporin 3 found in the basal cell layer of the epidermis) gene expression when they were applied for 24 h in a melanoma cell culture. AQP3 was pointed to as important marker in cancer.

VIII. Comparison of application of SFN non encapsulated and encapsulated SFN in broccoli membrane vesicles to melanoma cell culture (cell line SK-MEL-28) revealed better delivery of SFN into the cells when the compound was applied in the encapsulated form.

Objective 3: to determine the anti-inflammatory potential of broccoli membrane vesicles and encapsulated SFN in a human macrophage-like in vitro cell model under both normal and inflammatory conditions (Chapter III).

IX. Microsomal fraction vesicles from broccoli leaves were standardized through filtration and a homogeneous size about 200 nm was obtained, which did not change when SFN was encapsulated.

X. An *in vitro* release assay determined that the delivery of SFN encapsulated in broccoli membrane vesicles occurred in two phases. First, SFN was released more rapidly, possibly through lipid membrane, and then in a slower and more controlled manner, which could be due to interaction with membrane proteins.

XI. An *in silico* study determined a specific interaction between SFN and plant PIP aquaporins present in membrane vesicles via the conserved NPA motifs of aquaporins, which may enhance stabilization of the compound in the vesicles.

XII. The anti-inflammatory potential of SFN-loaded broccoli membrane vesicles in a human macrophage-like cell model *in vitro* (differentiated HL-60 cells) due to a significant inhibition of the inflammatory cytokines TNF-α, IL-6, and IL-1β levels with very low cytotoxicity.

GENERAL BIBLIOGRAPHY

General bibliography

Akuma P, Okagu OD, Udenigwe CC (2019) Naturally Occurring Exosome Vesicles as Potential Delivery Vehicle for Bioactive Compounds. Front Sustain Food Syst **3**: 23

Al-Hakkani MF (2020) Biogenic copper nanoparticles and their applications: A review. SN Appl Sci **2**: 505

Ali NB, Abdull Razis AF, Ooi DJ, Chan KW, Ismail N, Foo JB (2022) Theragnostic Applications of Mammal and Plant-Derived Extracellular Vesicles: Latest Findings, Current Technologies, and Prospects. Molecules **27**: 3941

Allahou LW, Madani SY, Seifalian A (2021) Investigating the Application of Liposomes as Drug Delivery Systems for the Diagnosis and Treatment of Cancer. Int J Biomater **2021**: 3041969

Alleva K, Niemietz CM, Sutka M, Maurel C, Parisi M, Tyerman SD, Amodeo G (2006) Plasma membrane of Beta vulgaris storage root shows high water channel activity regulated by cytoplasmic pH and a dual range of calcium concentrations. J Exp Bot **57**: 609–621

Alonso J, Khurshid H, Devkota J, Nemati Z, Khadka NK, Srikanth H, Pan J, Phan MH (2016) Superparamagnetic nanoparticles encapsulated in lipid vesicles for advanced magnetic hyperthermia and biodetection. J Appl Phys **119**: 083904

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: Problems and promises. Mol Pharm **4**: 807–818

Anderson RGW, Jacobson K (2002) A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. Science **296**: 1821 – 1825

Aquilano K, Ceci V, Gismondi A, De Stefano S, Iacovelli F, Faraonio R, Di Marco G, Poerio N, Minutolo A, Minopoli G, et al (2019) Adipocyte metabolism is improved by TNF receptor-targeting small RNAs identified from dried nuts. Commun Biol **2**: 317

Assadpour E, Mahdi Jafari S (2019) A systematic review on nanoencapsulation of food bioactive ingredients and nutraceuticals by various nanocarriers. Crit Rev Food Sci Nutr **59**: 3129–3151

Aziz ZAA, Mohd-Nasir H, Ahmad A, Siti SH, Peng WL, Chuo SC, Khatoon A, Umar K, Yaqoob AA, Mohamad Ibrahim MN (2019) Role of Nanotechnology for Design and Development of Cosmeceutical: Application in Makeup and Skin Care. Front Chem **7**: 739

Baenas N, Piegholdt S, Schloesser A, Moreno DA, García-Viguera C, Rimbach G, Wagner AE (2016) Metabolic activity of radish sprouts derived isothiocyanates in drosophila melanogaster. Int J Mol Sci **17**: 251

Bangham AD, Standish MM, Watkins JC (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol **13**: 238–252

Basyuni M, Baba S, Kinjo Y, Oku H (2012) Salinity increases the triterpenoid content of a salt secretor and a non-salt secretor mangrove. Aquat Bot **97**: 17–23

Bellati J, Champeyroux C, Hem S, Rofidal V, Krouk G, Maurel C, Santoni V (2016) Novel Aquaporin Regulatory Mechanisms Revealed by Interactomics. Mol Cell Proteomics **15**: 3473–3487

Belugin B V., Zhestkova IM, Trofimova MS (2011) Affinity of PIP-aquaporins to sterol-enriched domains in plasma membrane of the cells of etiolated pea seedlings. Biochem Suppl Ser A Membr Cell Biol **5**: 56–63

Blažević I, Montaut S, Burčul F, Olsen CE, Burow M, Rollin P, Agerbirk N (2020) Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants. Phytochemistry **169**: 112100

Bompard J, Rosso A, Brizuela L, Mebarek S, Blum LJ, Trunfio-Sfarghiu AM, Lollo G, Granjon T, Girard-Egrot A, Maniti O (2020) Membrane Fluidity as a New Means to Selectively Target Cancer Cells with Fusogenic Lipid Carriers. Langmuir **36**: 5134–5144

Cai X, Fang Z, Dou J, Yu A, Zhai G (2013) Bioavailability of Quercetin: Problems and Promises. Curr Med Chem **20**: 2572–2582

Caldeira C, De Laurentiis V, Corrado S, van Holsteijn F, Sala S (2019) Quantification of food waste per product group along the food supply chain in the European Union: a mass flow analysis. Resour Conserv Recycl **149**: 479–488

Cano-Sarabia M, Maspoch D (2015) Nanoencapsulation. Encycl. Nanotechnol. pp 1–16

Carter P, Narasimhan B, Wang Q (2019) Biocompatible nanoparticles and vesicular systems in transdermal drug delivery for various skin diseases. Int J Pharm **555**: 49–62

Carugo D, Bottaro E, Owen J, Stride E, Nastruzzi C (2016) Liposome production by microfluidics: Potential and limiting factors. Sci Rep **6**: 25876

Carvajal M, Cooke DT, Clarkson DT (1996) Plasma membrane fluidity and hydraulic conductance in wheat roots: Interactions between root temperature and nitrate or phosphate deprivation. Plant, Cell Environ **19**: 1110–1114

Carvajal M, García-Viguera C, Moreno DA, Martínez-Ballesta MC (2011) Method for obtaining plasma membrane vesicles extracted from plants enriched in membrane transport proteins, and uses thereof. Patent EP2716280

Casado-Vela J, Muries B, Carvajal M, Iloro I, Elortza F, Martínez-Ballesta MC (2010) Analysis of root plasma membrane aquaporins from brassica oleracea: Post-translational modifications, de novo sequencing and detection of isoforms by high resolution mass spectrometry. J Proteome Res **9**: 3479–3494

Chalbi N, Martínez-Ballesta MC, Youssef N Ben, Carvajal M (2015) Intrinsic stability of Brassicaceae plasma membrane in relation to changes in proteins and lipids as a response to salinity. J Plant Physiol **175**: 148–156

Chaudhary H, Kohli K, Kumar V (2014) A novel nano-carrier transdermal gel against inflammation. Int J Pharm **465**: 175–186

Chaumont F, Tyerman SD (2014) Aquaporins: Highly regulated channels controlling plant water relations. Plant Physiol **164**: 1600–1618

Chen X, Zhou Y, Yu J (2019) Exosome-like Nanoparticles from Ginger Rhizomes Inhibited NLRP3 Inflammasome Activation. Mol Pharm **16**: 2690–2699

Cheng YM, Tsai CC, Hsu YC (2016) Sulforaphane, a dietary isothiocyanate, induces G2/M arrest in cervical cancer cells through cyclinB1 downregulation and GADD45β/CDC2 association. Int J Mol Sci **17**: 1–13

Chevalier AS, Chaumont F (2015) Trafficking of plant plasma membrane aquaporins: Multiple regulation levels and complex sorting signals. Plant Cell Physiol **56**: 819–829

Ciancaglini P, Simão AMS, Bolean M, Millán JL, Rigos CF, Yoneda JS, Colhone MC, Stabeli RG (2012) Proteoliposomes in nanobiotechnology. Biophys Rev **4**: 67–81

Cocetta G, Mishra S, Raffaelli A, Ferrante A (2018) Effect of heat root stress and high salinity on glucosinolates metabolism in wild rocket. J Plant Physiol **231**: 261–270

Dad HA, Gu TW, Zhu AQ, Huang LQ, Peng LH (2021) Plant Exosome-like Nanovesicles: Emerging Therapeutics and Drug Delivery Nanoplatforms. Mol Ther **29**: 13–31

Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, Khorasani S, Mozafari MR (2018) Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics **10**: 57

Danielson JÅH, Johanson U (2008) Unexpected complexity of the Aquaporin gene family in the moss Physcomitrella patens. BMC Plant Biol **8**: 45

Daraee H, Etemadi A, Kouhi M, Alimirzalu S, Akbarzadeh A (2016) Application of liposomes in medicine and drug delivery. Artif Cells, Nanomedicine Biotechnol **44**: 381–391

Deng Z, Rong Y, Teng Y, Mu J, Zhuang X, Tseng M, Samykutty A, Zhang L, Yan J, Miller D, et al (2017) Broccoli-Derived Nanoparticle Inhibits Mouse Colitis by Activating Dendritic Cell AMP-Activated Protein Kinase. Mol Ther **25**: 1641–1654

Dinkova-Kostova AT, Kostov R V. (2012) Glucosinolates and isothiocyanates in health and disease. Trends Mol Med **18**: 337–347

Do DP, Pai SB, Rizvi SAA, D'Souza MJ (2010) Development of sulforaphane-encapsulated microspheres for cancer epigenetic therapy. Int J Pharm **386**: 114–121

Dolai J, Mandal K, Jana NR (2021) Nanoparticle Size Effects in Biomedical Applications. ACS Appl Nano Mater **4**: 6471–6496

Domínguez-Perles R, Martínez-Ballesta MC, Carvajal M, García-Viguera C, Moreno DA (2010) Broccoli-derived by-products - a promising source of bioactive ingredients. J Food Sci **75**: 383–392

Drabińska N, Ciska E, Szmatowicz B, Krupa-Kozak U (2018) Broccoli by-products improve the nutraceutical potential of gluten-free mini sponge cakes. Food Chem **267**: 170–177

Duhan JS, Kumar R, Kumar N, Kaur P, Nehra K, Duhan S (2017) Nanotechnology: The new perspective in precision agriculture. Biotechnol Reports **15**: 11–23

Elahi N, Kamali M, Baghersad MH (2018) Recent biomedical applications of gold nanoparticles: A review. Talanta **184**: 537–556

Elias PM (2005) Stratum corneum defensive functions: An integrated view. J Invest Dermatol **125**: 183–200

Engelman DM (2005) Membranes are more mosaic than fluid. Nature **438**: 578–580

Enriquez GG, Rizvi SAA, D'Souza MJ, Do DP (2013) Formulation and evaluation of drug-loaded targeted magnetic microspheres for cancer therapy. Int J Nanomedicine **8**: 1393–1402

Estephan M, El Kurdi R, Patra D (2022) Curcumin-embedded DBPC liposomes coated with chitosan layer as a fluorescence nanosensor for the selective detection of ribonucleic acid. Luminescence **37**: 422–430

Van Eylen D, Oey I, Hendrickx M, Van Loey A (2007) Kinetics of the stability of broccoli (Brassica oleracea Cv. Italica) myrosinase and isothiocyanates in broccoli juice during pressure/temperature treatments. J Agric Food Chem **55**: 2163–2170

Farkhani SM, Valizadeh A, Karami H, Mohammadi S, Sohrabi N, Badrzadeh F (2014) Cell penetrating peptides: Efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules. Peptides **57**: 78–94

Fatima U, Ahmad F, Ramzan M, Aziz S, Tariq M, Iqbal HMN, Imran M (2021) Catalytic transformation of Brassica nigra oil into biodiesel using in-house engineered green catalyst: Development and characterization. Clean Technol Environ Policy 1–11

FDA (2016) Is It a Cosmetic, a Drug, or Both? (Or Is It Soap?). U.S. Food Drug Adm.,

Feng X, Xu W, Li Z, Song W, Ding J, Chen X (2019) Immunomodulatory Nanosystems. Adv Sci **6**: 1900101

Fernández-Ochoa Á, Leyva-Jiménez FJ, Pimentel-Moral S, del Carmen Villegas-Aguilar M, Alañón ME, Segura-Carretero A, de la Luz Cádiz-Gurrea M (2021) Revalorisation of Agro-Industrial Wastes into High Value-Added Products. Adv. Sci. Technol. Innov. pp 229–245

Finbloom JA, Sousa F, Stevens MM, Desai TA (2020) Engineering the drug carrier biointerface to overcome biological barriers to drug delivery. Adv Drug Deliv Rev **167**: 89–108

Fink M, Feller C, Scharpf HC, Weier U, Maync A, Ziegler J, Paschold PJ, Strohmeyer K (1999) Nitrogen, phosphorus, potassium and magnesium contents of field vegetables - Recent data for fertiliser recommendations and nutrient balances. J Plant Nutr Soil Sci **162**: 71–73

Finn RN, Cerdá J (2015) Evolution and functional diversity of aquaporins. Biol Bull **229**: 6–23

Fox CB, Kim J, Le L V., Nemeth CL, Chirra HD, Desai TA (2015) Micro/nanofabricated platforms for oral drug delivery. J Control Release **219**: 431–444

Frank LA, Contri R V., Beck RCR, Pohlmann AR, Guterres SS (2015) Improving drug biological effects by encapsulation into polymeric nanocapsules. Wiley Interdiscip Rev Nanomedicine Nanobiotechnology **7**: 623–639

Fuentes F, Paredes-Gonzalez X, Kong ANT (2015) Dietary Glucosinolates Sulforaphane, Phenethyl Isothiocyanate, Indole-3-Carbinol/3,3′-Diindolylmethane: Antioxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and In Vivo Cancer Chemopreventive Efficacy. Curr Pharmacol Reports **1**: 179–196

Fujita D, Arai T, Komori H, Shirasaki Y, Wakayama T, Nakanishi T, Tamai I (2018) Apple-Derived Nanoparticles Modulate Expression of Organic-Anion-Transporting Polypeptide (OATP) 2B1 in Caco-2 Cells. Mol Pharm **15**: 5772–5780

Fujiwara N, Kobayashi K (2005) Macrophages in inflammation. Curr Drug Targets Inflamm Allergy **4**: 281–286

Garaeva L, Kamyshinsky R, Kil Y, Varfolomeeva E, Verlov N, Komarova E, Garmay Y, Landa S, Burdakov V, Myasnikov A, et al (2021) Delivery of functional exogenous

proteins by plant-derived vesicles to human cells in vitro. Sci Rep **11**: 1–12

Garcia-Ibañez P, Moreno DA, Carvajal M (2022) Nanoencapsulation of Bimi® extracts increases its bioaccessibility after in vitro digestion and evaluation of its activity in hepatocyte metabolism. Food Chem **385**: 132680

Garcia-Ibañez P, Roses C, Agudelo A, Milagro FI, Barceló AM, Viadel B, Nieto JA, Moreno DA, Carvajal M (2021) The influence of red cabbage extract nanoencapsulated with brassica plasma membrane vesicles on the gut microbiome of obese volunteers. Foods **10**: 1038

García-Ibañez P, Yepes-Molina L, Ruiz-Alcaraz AJ, Martínez-Esparza M, Moreno DA, Carvajal M, García-Peñarrubia P (2020) Brassica bioactives could ameliorate the chronic inflammatory condition of endometriosis. Int J Mol Sci **21**: 9397

Gardiner C, Vizio D Di, Sahoo S, Théry C, Witwer KW, Wauben M, Hill AF (2016) Techniques used for the isolation and characterization of extracellular vesicles: Results of a worldwide survey. J Extracell Vesicles **5**: 32945

Gerbeau P, Amodeo G, Henzler T, Santoni V, Ripoche P, Maurel C (2002) The water permeability of Arabidopsis plasma membrane is regulated by divalent cations and pH. Plant J **30**: 71–81

Giglione C, Fieulaine S, Meinnel T (2015) N-terminal protein modifications: Bringing back into play the ribosome. Biochimie **114**: 134–146

Gillet A, Compère P, Lecomte F, Hubert P, Ducat E, Evrard B, Piel G (2011) Liposome surface charge influence on skin penetration behaviour. Int J Pharm **411**: 223–231

González-Mariscal L, Nava P, Hernández S (2005) Critical role of tight junctions in drug delivery across epithelial and endothelial cell layers. J Membr Biol **207**: 55–68

Gonzalez Gomez A, Hosseinidoust Z (2020) Liposomes for Antibiotic Encapsulation and Delivery. ACS Infect Dis **6**: 896–908

Guidotti G (1972) Membrane proteins. Annu Rev Biochem **41**: 731–752

Gupta A, Mumtaz S, Li CH, Hussain I, Rotello VM (2019) Combatting antibiotic-resistant bacteria using nanomaterials. Chem Soc Rev **48**: 415–427

Hachez C, Laloux T, Reinhardt H, Cavez D, Degand H, Grefen C, De Rycke R, Inzé D, Blatt MR, Russinova E, et al (2014a) Arabidopsis SNAREs SYP61 and SYP121 coordinate the trafficking of plasma membrane aquaporin PIP2;7 to modulate the cell membrane water permeability. Plant Cell **26**: 3132–3147

Hachez C, Veljanovski V, Reinhardt H, Guillaumot D, Vanhee C, Chaumont F, Batoko H (2014b) The Arabidopsis Abiotic Stress-Induced Tspo-Related Protein Reduces Cell-Surface Expression of the Aquaporin PIP2;7 through Protein-Protein Interactions and Autophagic Degradation. Plant Cell **26**: 4974–4990

Heisig M, Lieckfeldt R, Wittum G, Mazurkevich G, Lee G (1996) Non steady-state descriptions of drug permeation through stratum corneum. I. The biphasic brick-and-mortar model. Pharm Res **13**: 421–426

Heo DN, Ko WK, Bae MS, Lee JB, Lee DW, Byun W, Lee CH, Kim EC, Jung BY, Kwon IK (2014) Enhanced bone regeneration with a gold nanoparticle-hydrogel complex. J Mater Chem B **2**: 1584–1593

Ho AW, Kupper TS (2019) T cells and the skin: from protective immunity to inflammatory skin disorders. Nat Rev Immunol **19**: 490–502

Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, Soteropoulos P, Tolias P, Hart RP, Kong ANT (2004) In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther **310**: 263–271

Huynh NT, Roger E, Lautram N, Benoît JP, Passirani C (2010) The rise and rise of stealth nanocarriers for cancer therapy: Passive versus active targeting. Nanomedicine **5**: 1415–1433

Italian Government (2019) A new bioeconomy strategy for a sustainable Italy. Eur Comm Press Release 716–734

Itel F, Al-Samir S, Öberg F, Chami M, Kumar M, Supuran CT, Deen PMT, Meier W, Hedfalk K, Gros G, et al (2012) CO2 permeability of cell membranes is regulated by membrane cholesterol and protein gas channels. FASEB J **26**: 5182–5191

Jeffery EH, Araya M (2009) Physiological effects of broccoli consumption. Phytochem Rev **8**: 283–298

Jeffery EH, Brown AF, Kurilich AC, Keck AS, Matusheski N, Klein BP, Juvik JA (2003) Variation in content of bioactive components in broccoli. J Food Compos Anal **16**: 323–330

Jin Y, Wang M, Rosen RT, Ho CT (1999) Thermal degradation of sulforaphane in aqueous solution. J Agric Food Chem **47**: 3121–3123

Ju S, Mu J, Dokland T, Zhuang X, Wang Q, Jiang H, Xiang X, Deng Z Bin, Wang B, Zhang L, et al (2013) Grape exosome-like nanoparticles induce intestinal stem cells and protect mice from DSS-induced colitis. Mol Ther **21**: 1345–1357

Kai L, Kaldenhoff R (2014) A refined model of water and CO2 membrane diffusion: Effects and contribution of sterols and proteins. Sci Rep **4**: 6665

Kameli N, Dragojlovic-kerkache A, Savelkoul P, Stassen FR (2021) Plant-derived extracellular vesicles: Current findings, challenges, and future applications. Membranes (Basel) **11**: 411

Kanfer I, Shargel L (2020) Approved Drug Products with Therapeutic Equivalence Evaluations (The Orange Book). Generic Drug Prod. Dev. pp 36–51

Kelly PJ, Bones A, Rossiter JT (1998) Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of Brassica juncea. Planta **206**: 370–377

Kim K, Yoo HJ, Jung JH, Lee R, Hyun JK, Park JH, Na D, Yeon JH (2020) Cytotoxic effects of plant sap-derived extracellular vesicles on various tumor cell types. J Funct Biomater **11**: 22

Kim MK, Choi YC, Cho SH, Choi JS, Cho YW (2021) The Antioxidant Effect of Small Extracellular Vesicles Derived from Aloe vera Peels for Wound Healing. Tissue Eng Regen Med **18**: 561–571

King LS, Kozono D, Agre P (2004) From structure to disease: The evolving tale of aquaporin biology. Nat Rev Mol Cell Biol **5**: 687–698

Krishnan V, Mitragotri S (2020) Nanoparticles for topical drug delivery: Potential for skin cancer treatment. Adv Drug Deliv Rev **153**: 87–108

Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ, Robinson C V (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature **510**: 172–5

Lai SK, Wang YY, Hanes J (2009) Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev **61**: 158–171

Lasic DD (1997) Recent developments in medical applications of liposomes: Sterically stabilized liposomes in cancer therapy and gene delivery in vivo. J Control Release **48**: 203–222

Lee HJ, Hong YJ, Kim M (2021) Angiogenesis in chronic inflammatory skin disorders. Int J Mol Sci **22**: 12035

Lefebvre B, Furt F, Hartmann M-A, Michaelson L V., Carde J-P, Sargueil-Boiron F, Rossignol M, Napier JA, Cullimore J, Bessoule J-J, et al (2007) Characterization of Lipid Rafts from Medicago truncatula Root Plasma Membranes: A Proteomic Study Reveals the Presence of a Raft-Associated Redox System. PLANT Physiol **144**: 402–418

Leiter U, Keim U, Garbe C (2020) Epidemiology of skin cancer: Update 2019. Adv. Exp. Med. Biol. pp 123–139

Li P, Kaslan M, Lee SH, Yao J, Gao Z (2017) Progress in exosome isolation techniques. Theranostics **7**: 789–804

Li X, Wang X, Yang Y, Li R, He Q, Fang X, Luu D-T, Maurel C, Lin J (2011) Single-Molecule Analysis of PIP2;1 Dynamics and Partitioning Reveals Multiple Modes of Arabidopsis Plasma Membrane Aquaporin Regulation. Plant Cell **23**: 3780–3797

Liljenberg CS (1992) The effects of water deficit stress on plant membrane lipids. Prog Lipid Res **31**: 335–343

Liu NJ, Wang N, Bao JJ, Zhu HX, Wang LJ, Chen XY (2020) Lipidomic Analysis Reveals the Importance of GIPCs in Arabidopsis Leaf Extracellular Vesicles. Mol Plant **13**: 1523–1532

Liu P, Atkinson SJ, Akbareian SE, Zhou Z, Munsterberg A, Robinson SD, Bao Y (2017) Sulforaphane exerts anti-angiogenesis effects against hepatocellular carcinoma through inhibition of STAT3/HIF-1α/VEGF signalling. Sci Rep **7**: 12651

López-Pérez L, Martínez-Ballesta M del C, Maurel C, Carvajal M (2009) Changes in plasma membrane lipids, aquaporins and proton pump of broccoli roots, as an adaptation mechanism to salinity. Phytochemistry **70**: 492–500

Lu M, Zhao X, Xing H, Xun Z, Yang T, Cai C, Wang D, Ding P (2018) Liposome-chaperoned cell-free synthesis for the design of proteoliposomes: Implications for therapeutic delivery. Acta Biomater **76**: 1–20

Luu DT, Maurel C (2013) Aquaporin Trafficking in Plant Cells: An Emerging Membrane-Protein Model. Traffic **14**: 629–635

Maja L, Željko K, Mateja P (2020) Sustainable technologies for liposome preparation. J Supercrit Fluids **165**: 104984

Martina MS, Fortin JP, Ménager C, Clément O, Barratt G, Grabielle-Madelmont C, Gazeau F, Cabuil V, Lesieur S (2005) Generation of superparamagnetic liposomes revealed as highly efficient MRI contrast agents for in vivo imaging. J Am Chem Soc **127**: 10676–10685

Martínez-Ballesta M del C, Carvajal M (2016) Mutual interactions between aquaporins and membrane components. Front Plant Sci **7**: 1322

Martínez-Ballesta M del C, Moreno DA, Carvajal M (2013) The physiological importance of glucosinolates on plant response to abiotic stress in Brassica. Int J Mol Sci **14**: 11607–11625

Martínez Ballesta MC, García-Gomez P, Yepes-Molina L, Guarnizo AL, Teruel JA, Carvajal M (2018) Plasma membrane aquaporins mediates vesicle stability in broccoli. PLoS One **13**: 1–19

Martínez Ballesta MC, Pérez-Sánchez H, Moreno DA, Carvajal M (2016) Plant plasma membrane aquaporins in natural vesicles as potential stabilizers and carriers of glucosinolates. Colloids Surfaces B Biointerfaces **143**: 318–326

Maurel C, Boursiac Y, Luu DT, Santoni V, Shahzad Z, Verdoucq L (2015) Aquaporins in plants. Physiol Rev **95**: 1321–1358

Maurel C, Reizer J, Schroeder JI, Chrispeels MJ (1993) The vacuolar membrane protein gamma-TIP creates water specific channels in Xenopus oocytes. EMBO J **12**: 2241–2247

Minami A, Fujiwara M, Furuto A, Fukao Y, Yamashita T, Kamo M, Kawamura Y, Uemura M (2009) Alterations in detergent-resistant plasma membrane microdomains in Arabidopsis thaliana during cold acclimation. Plant Cell Physiol **50**: 341–359

Mitchell MJ, Billingsley MM, Haley RM, Wechsler ME, Peppas NA, Langer R (2021) Engineering precision nanoparticles for drug delivery. Nat Rev Drug Discov **20**: 101–124

Mittal S, Saluja D (2015) Protein Post-translational Modifications: Role in Protein Structure, Function and Stability. *In* LR Singh, TA Dar, P Ahmad, eds, Proteostasis Chaperone Surveill. Springer India, New Delhi, pp 25–37

Mohanpuria P, Rana NK, Yadav SK (2008) Biosynthesis of nanoparticles: Technological concepts and future applications. J Nanoparticle Res **10**: 507–517

Moreno DA, Carvajal M, López-Berenguer C, García-Viguera C (2006) Chemical and biological characterisation of nutraceutical compounds of broccoli. J Pharm Biomed Anal **41**: 1508–1522

Mu J, Zhuang X, Wang Q, Jiang H, Deng Z Bin, Wang B, Zhang L, Kakar S, Jun Y, Miller D, et al (2014) Interspecies communication between plant and mouse gut host cells through edible plant derived exosome-like nanoparticles. Mol Nutr Food Res **58**: 1561–1573

Mulcahy LA, Pink RC, Carter DRF (2014) Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles **3**: 24641

Murata K, Mitsuoka K, Hiral T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y (2000) Structural determinants of water permeation through aquaporin-1. Nature **407**: 599–605

Muries B, Carvajal M, Martínez-Ballesta MC (2013) Response of three broccoli cultivars to salt stress, in relation to water status and expression of two leaf aquaporins. Planta **237**: 1297–1310

Muries B, Mohamed F, Carvajal M, Martínez-Ballesta MC (2011) Identification and differential induction of the expression of aquaporins by salinity in broccoli plants. Mol Biosyst **7**: 1322–1335

Nemati M, Singh B, Mir RA, Nemati M, Babaei A, Ahmadi M, Rasmi Y, Golezani AG,

Rezaie J (2022) Plant-derived extracellular vesicles: a novel nanomedicine approach with advantages and challenges. Cell Commun Signal **20**: 69

Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, et al (2017) Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet **390**: 2769–2778

Pachuau L, Laldinchhana, Roy PK, Zothantluanga JH, Ray S, Das S (2021) Encapsulation of Bioactive Compound and Its Therapeutic Potential. Bioact. Nat. Prod. Pharm. Appl. pp 687–714

Panche AN, Diwan AD, Chandra SR (2016) Flavonoids: An overview. J Nutr Sci **5**: E47

Patra JK, Das G, Fraceto LF, Campos EVR, Rodriguez-Torres MDP, Acosta-Torres LS, Diaz-Torres LA, Grillo R, Swamy MK, Sharma S, et al (2018) Nano based drug delivery systems: recent developments and future prospects. J Nanobiotechnology **16**: 71

Pérez-Bermúdez P, Blesa J, Soriano JM, Marcilla A (2017) Extracellular vesicles in food: Experimental evidence of their secretion in grape fruits. Eur J Pharm Sci **98**: 40–50

Peskan T, Westermann M, Oelmüller R (2000) Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. Eur J Biochem **267**: 6989 – 6995

Pieniazek M, Matkowski R, Donizy P (2018) Macrophages in skin melanoma-the key element in melanomagenesis. Oncol Lett **15**: 5399–5404

Poh AR, Ernst M (2018) Targeting macrophages in cancer: From bench to bedside. Front Oncol **8**: 49

Polevoda B, Sherman F (2000) Nalpha -terminal acetylation of eukaryotic proteins. J Biol Chem **275**: 36479–36482

Quirante-Moya S, García-Ibañez P, Quirante-Moya F, Villaño D, Moreno DA (2020) The role of Brassica bioactives on human health: Are we studying it the right way? Molecules **25**: 1591

Raimondo S, Naselli F, Fontana S, Monteleone F, Lo Dico A, Saieva L, Zito G, Flugy A, Manno M, Di Bella MA, et al (2015) Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. Oncotarget **6**: 19514–19527

Raval N, Maheshwari R, Kalyane D, Youngren-Ortiz SR, Chougule MB, Tekade RK (2018) Importance of physicochemical characterization of nanoparticles in pharmaceutical product development. Basic Fundam. Drug Deliv. pp 369–400

Rios JJ, Garcia-Ibañez P, Carvajal M (2019) The use of biovesicles to improve the efficiency of Zn foliar fertilization. Colloids Surfaces B Biointerfaces **173**: 899–905

Rios JJ, Yepes-Molina L, Martinez-Alonso A, Carvajal M (2020) Nanobiofertilization as a novel technology for highly efficient foliar application of Fe and B in almond trees. R Soc Open Sci **7**: 200905

Rizwan M, Ali S, Zia ur Rehman M, Rinklebe J, Tsang DCW, Bashir A, Maqbool A, Tack FMG, Ok YS (2018) Cadmium phytoremediation potential of Brassica crop species: A review. Sci Total Environ **631**–**632**: 1175–1191

Roursgaard M, Knudsen KB, Northeved H, Persson M, Christensen T, Kumar PEK, Permin A, Andresen TL, Gjetting T, Lykkesfeldt J, et al (2016) In vitro toxicity of cationic micelles and liposomes in cultured human hepatocyte (HepG2) and lung epithelial (A549) cell lines. Toxicol Vitr **36**: 164–171

Rout GK, Shin H-S, Gouda S, Sahoo S, Das G, Fraceto LF, Patra JK (2018) Current advances in nanocarriers for biomedical research and their applications. Artif Cells, Nanomedicine, Biotechnol **46**: 1053–1062

Ruhee RT, Suzuki K (2020) The integrative role of sulforaphane in preventing inflammation, oxidative stress and fatigue: A review of a potential protective phytochemical. Antioxidants **9**: 1–13

Seneviratne R, Khan S, Moscrop E, Rappolt M, Muench SP, Jeuken LJC, Beales PA (2018) A reconstitution method for integral membrane proteins in hybrid lipid-polymer vesicles for enhanced functional durability. Methods **147**: 142–149

Shashanka R, Kumara Swamy BE (2020) Biosynthesis of silver nanoparticles using leaves of Acacia melanoxylon and their application as dopamine and hydrogen peroxide sensors. Phys Chem Res **8**: 1–18

Shishir MRI, Xie L, Sun C, Zheng X, Chen W (2018) Advances in micro and nano-encapsulation of bioactive compounds using biopolymer and lipid-based transporters. Trends Food Sci Technol **78**: 34–60

Silva C, Aranda FJ, Ortiz A, Carvajal M, Martlnez V, Teruel JA (2007) Root Plasma Membrane Lipid Changes in Relation to Water Transport in Pepper : a Response to NaCI and CaCI2 Treatment. **50**: 650–657

Silva C, Aranda FJ, Ortiz A, Martínez V, Carvajal M, Teruel JA (2011) Molecular aspects of the interaction between plants sterols and DPPC bilayers. An experimental and theoretical approach. J Colloid Interface Sci **358**: 192–201

Simões MCF, Sousa JJS, Pais AACC (2015) Skin cancer and new treatment perspectives: A review. Cancer Lett **357**: 8–42

Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science (80-) **175**: 720–731

Singh RP (2021) Recent Trends, Prospects, and Challenges of Nanobiosensors in Agriculture. Biosens. Agric. Recent Trends Futur. Perspect. pp 3–13

Sinico C, Manconi M, Peppi M, Lai F, Valenti D, Fadda AM (2005) Liposomes as carriers for dermal delivery of tretinoin: In vitro evaluation of drug permeation and vesicle-skin interaction. J Control Release **103**: 123–136

Soenen SJ, Rivera-Gil P, Montenegro JM, Parak WJ, De Smedt SC, Braeckmans K (2011) Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation. Nano Today **6**: 446–465

Solomon D, Gupta N, Mulla NS, Shukla S, Guerrero YA, Gupta V (2017) Role of In Vitro Release Methods in Liposomal Formulation Development: Challenges and Regulatory Perspective. AAPS J **19**: 1669–1681

Soto G, Alleva K, Amodeo G, Muschietti J, Ayub ND (2012) New insight into the evolution of aquaporins from flowering plants and vertebrates: Orthologous identification and functional

transfer is possible. Gene **503**: 165–176

Sun G, Chung TS, Jeyaseelan K, Armugam A (2013) Stabilization and immobilization of aquaporin reconstituted lipid vesicles for water purification. Colloids Surfaces B Biointerfaces **102**: 466–471

Sundaram K, Miller DP, Kumar A, Teng Y, Sayed M, Mu J, Lei C, Sriwastva MK, Zhang L, Jun Y, et al (2019) Plant-Derived Exosomal Nanoparticles Inhibit Pathogenicity of Porphyromonas gingivalis. iScience **21**: 308–327

Tapken W, Murphy AS (2015) Membrane nanodomains in plants: Capturing form, function, and movement. J Exp Bot **66**: 1573–1586

Teissier É, Pécheur EI (2007) Lipids as modulators of membrane fusion mediated by viral fusion proteins. Eur Biophys J **36**: 887–899

Tenchov R, Bird R, Curtze AE, Zhou Q (2021) Lipid Nanoparticles from Liposomes to mRNA Vaccine Delivery, a Landscape of Research Diversity and Advancement. ACS Nano **15**: 16982–17015

Teng Y, Ren Y, Sayed M, Hu X, Lei C, Kumar A, Hutchins E, Mu J, Deng Z, Luo C, et al (2018) Plant-Derived Exosomal MicroRNAs Shape the Gut Microbiota. Cell Host Microbe **24**: 637-652.e8

Terstappen GC, Meyer AH, Bell RD, Zhang W (2021) Strategies for delivering therapeutics across the blood–brain barrier. Nat Rev Drug Discov **20**: 362–383

Tian G, Li Y, Yuan Q, Cheng L, Kuang P, Tang P (2015) The stability and degradation kinetics of Sulforaphene in microcapsules based on several biopolymers via spray drying. Carbohydr Polym **122**: 5–10

Tian S, Liu X, Lei P, Zhang X, Shan Y (2018) Microbiota: a mediator to transform glucosinolate precursors in cruciferous vegetables to the active isothiocyanates. J Sci Food Agric **98**: 1255–1260

Tiwari N, Osorio-Blanco ER, Sonzogni A, Esporrín-Ubieto D, Wang H, Calderón M (2022) Nanocarriers for Skin Applications: Where Do We Stand? Angew Chemie - Int Ed **61**: e202107960

Tong J, Briggs MM, McIntosh TJ (2012) Water permeability of aquaporin-4 channel depends on bilayer composition, thickness, and elasticity. Biophys J **103**: 1899–1908

Törnroth-Horsefield S, Wang Y, Hedfalk K, Johanson U, Karlsson M, Tajkhorshid E, Neutze R, Kjellbom P (2006) Structural mechanism of plant aquaporin gating. Nature **439**: 688–694

Uematsu M, Shimizu T (2021) Raman microscopy-based quantification of the physical properties of intracellular lipids. Commun Biol **4**: 1176

Urzì O, Gasparro R, Ganji NR, Alessandro R, Raimondo S (2022) Plant-RNA in Extracellular Vesicles: The Secret of Cross-Kingdom Communication. Membranes (Basel) **12**: 352

Vaishnav RA, Liu R, Chapman J, Roberts AM, Ye H, Rebolledo-Mendez JD, Tabira T, Fitzpatrick AH, Achiron A, Running MP, et al (2013) Aquaporin 4 molecular mimicry and implications for neuromyelitis optica. J Neuroimmunol **260**: 92–98

Vallar S, Houivet D, El Fallah J, Kervadec D, Haussonne JM (1999) Oxide slurries stability

and powders dispersion: Optimization with zeta potential and rheological measurements. J Eur Ceram Soc **19**: 1017–1021

Wang B, Zhuang X, Deng Z Bin, Jiang H, Mu J, Wang Q, Xiang X, Guo H, Zhang L, Dryden G, et al (2014) Targeted drug delivery to intestinal macrophages by bioactive nanovesicles released from grapefruit. Mol Ther **22**: 522–534

Wang H, Liang H, Yuan Q, Wang T (2011) A novel pH-sensitive microsphere composed of CM-chitosan and alginate for sulforaphane delivery. Mater. Sci. Forum. pp 539–547

Wang J, Feng L, Zhu Z, Zheng M, Wang D, Chen Z, Sun H (2015) Aquaporins as diagnostic and therapeutic targets in cancer: How far we are? J Transl Med **13**: 96

Wang J, Gu H, Yu H, Zhao Z, Sheng X, Zhang X (2012) Genotypic variation of glucosinolates in broccoli (Brassica oleracea var. italica) florets from China. Food Chem **133**: 735–741

Wang Q, Zhuang X, Mu J, Deng Z Bin, Jiang H, Xiang X, Wang B, Yan J, Miller D, Zhang HG (2013) Delivery of therapeutic agents by nanoparticles made of grapefruit-derived lipids. Nat Commun **4**: 1867

Wang Y, Wang J, Ma J, Zhou Y, Lu R (2022) Focusing on Future Applications and Current Challenges of Plant Derived Extracellular Vesicles. Pharmaceuticals **15**: 708

Wittstock U, Halkier BA (2002) Glucosinolate research in the Arabidopsis era. Trends Plant Sci **7**: 263–270

Xi H, Cun D, Xiang R, Guan Y, Zhang Y, Li Y, Fang L (2013) Intra-articular drug delivery from an optimized topical patch containing teriflunomide and lornoxicam for rheumatoid arthritis treatment: Does the topical patch really enhance a local treatment? J Control Release **169**: 73–81

Yang M, Liu X, Luo Q, Xu L, Chen F (2020) An efficient method to isolate lemon derived extracellular vesicles for gastric cancer therapy. J Nanobiotechnology **18**: 100

Yang R, Wei T, Goldberg H, Wang W, Cullion K, Kohane DS (2017) Getting Drugs Across Biological Barriers. Adv Mater. doi: 10.1002/adma.201606596

Yepes-Molina L, Carvajal M, Martínez-Ballesta MC (2020) Detergent resistant membrane domains in broccoli plasma membrane associated to the response to salinity stress. Int J Mol Sci **21**: 7694

Yepes-Molina L, Hernández JA, Carvajal M (2021) Nanoencapsulation of Pomegranate Extract to Increase Stability and Potential Dermatological Protection. Pharmaceutics **13**: 271

You JY, Kang SJ, Rhee WJ (2021) Isolation of cabbage exosome-like nanovesicles and investigation of their biological activities in human cells. Bioact Mater **6**: 4321–4332

Yu L, Deng Z, Liu L, Zhang W, Wang C (2020) Plant-Derived Nanovesicles: A Novel Form of Nanomedicine. Front Bioeng Biotechnol **8**: 584391

Yuanfeng W, Chengzhi L, Ligen Z, Juan S, Xinjie S, Yao Z, Jianwei M (2021) Approaches for enhancing the stability and formation of sulforaphane. Food Chem **345**: 128771

Zaghdoud C, Alcaraz-López C, Mota-Cadenas C, Martnez-Ballesta MDC, Moreno DA, Ferchichi A, Carvajal M (2012) Differential responses of two broccoli (Brassica oleracea L. var Italica) cultivars to salinity and nutritional quality improvement. Sci World J **2012**: 1–12

Zambrano V, Bustos R, Mahn A (2019) Insights about stabilization of sulforaphane through microencapsulation. Heliyon **5**: e02951

Zanotto-Filho A, Coradini K, Braganhol E, Schröder R, De Oliveira CM, Simões-Pires A, Battastini AMO, Pohlmann AR, Guterres SS, Forcelini CM, et al (2013) Curcumin-loaded lipid-core nanocapsules as a strategy to improve pharmacological efficacy of curcumin in glioma treatment. Eur J Pharm Biopharm **83**: 156–167

Zecha J, Gabriel W, Spallek R, Chang YC, Mergner J, Wilhelm M, Bassermann F, Kuster B (2022) Linking post-translational modifications and protein turnover by site-resolved protein turnover profiling. Nat Commun **13**: 165

Zhang L, He F, Gao L, Cong M, Sun J, Xu J, Wang Y, Hu Y, Asghar S, Hu L, et al (2021) Engineering exosome-like nanovesicles derived from Asparagus cochinchinensis can inhibit the proliferation of hepatocellular carcinoma cells with better safety profile. Int J Nanomedicine **16**: 1575–1586

Zhang Z, Yu Y, Zhu G, Zeng L, Xu S, Cheng H, Ouyang Z, Chen J, Pathak JL, Wu L, et al (2022) The Emerging Role of Plant-Derived Exosomes-Like Nanoparticles in Immune Regulation and Periodontitis Treatment. Front Immunol **13**: 896745

Zhong H, Chan G, Hu Y, Hu H, Ouyang D (2018) A Comprehensive Map of FDA-Approved Pharmaceutical Products. Pharmaceutics **10**: 263