A New Approach for Anti-Inflammatory Therapy : Exosomal Fusion of *Hydrangea Macrophylla* Leaf Extracellular Vesicles for Effective Drug Delivery

Ryu, Ji Soo[1,4]; Park, Hye Sun[2]; Kim, Mi Jung[3]; Jeong, En Young[3]; Hong, Kwan Soo[2]; Kang, Hee Cheol[3]; **Seo, Jae Yong**[1,5*];

[1] Innovation materials Lab., Cosmecca Korea Co., Ltd, Seongnam, Republic of Korea;

[2] Biopharmaceutical Research Center, Korea Basic Science Institute, Republic of Korea;

[3] Human & Microbiome Communicating Laboratory, GFC Co., Ltd., Hwaseong, Republic of Korea;

[4] Department of Biocosmetics, Sungkyunkwan University, Suwon, Republic of Korea;

[5] Department of Cosmetics Engineering, Konkuk University, Seoul, Republic of Korea;

* Seo, Jae Yong, Innovation materials Lab., Cosmecca Korea Co., Ltd, Seongnam, Republic of Korea, +82-31-784-6540, sjyb612@cosmecca.com

Abstract

This research introduces a novel hybrid extracellular vesicle (EV)/liposome system formed via vesicular membrane fusion between anti-inflammatory *Hydrangea macrophylla* leaf EVs (HML-EVs) and liposomes loaded with terpinen-4-ol and azelamide monoethanolamine. Utilizing a two-color fluorescence resonance energy transfer mechanism, we confirmed the successful fusion of HML-EVs with the terpinen-4-ol/azelamide monoethanolamine-loaded liposome membranes. This facilitated the scaled production of a stable hybrid HML-EV/liposome dispersion, measuring 100–200 nm in length. Our results demonstrate that the hybrid

EV/liposome system significantly inhibits apoptosis by reducing the production of nitric oxide and tumor necrosis factor-α. Additionally, the HML-EV/liposomes effectively suppress the synthesis of protease-activated receptor-2, interleukin-6, and thymic stromal lymphopoietin, showcasing their anti-inflammatory properties. These findings underscore the potential of the hybrid EV/liposome system developed in this study to mitigate skin irritation and enhance skin anti-inflammatory responses.

Keywords: hybrid exosomes, anti-inflammatory effect, *hydrangea macrophylla* leaf extracellular vesicles

Introduction.

Liposomes, thin bilayer membranes in an aqueous medium, primarily facilitate drug delivery via passive accumulation in specific tissues, unless they bear additional surface ligands. However, conventional liposomes are susceptible to degradation by pH, enzymes, and the immune system in biological environments, reducing their bioavailability [1,2]. In contrast, extracellular vesicles (EVs), secreted by all living cells, including animals, plants, and microbial cells, can efficiently transmit signals to target cells due to their ability to transport various secondary metabolites, membrane proteins, and genetic information (DNAs, RNAs, and proteins) [3,4]. Additionally, they exhibit high biocompatibility and cell penetration rates. Despite their utility, natural EVs suffer from low productivity [5], structural instability, and low drug-loading capacity, necessitating the development of advanced EV technologies.

Plant-derived EVs, extracted from various plants, edible vegetables, and fruits, are particularly promising due to their low cytotoxicity and immunogenicity compared to animal-derived EVs. Moreover, they offer economic feasibility as drug carriers because of their natural composition, origin, and extractability from cost-effective plant sources. Consequently, plant-based EVs are

considered valuable biological nanomaterials in the cosmetic and food industries [6]. Hybrid EVs, engineered by modifying EVs biologically or chemically, represent a new generation of biomaterials aiming to overcome the limitations of natural EVs and synthetic liposomes through mutual membrane fusion. Modifying the EV surface via liposomal membrane fusion is expected to reduce immunogenicity, thereby improving structural stability, drug loading, and delivery efficacy [7].

This study introduces a hybrid EV/liposome system employing membrane fusion technology to induce liposomal hybridization between plant-based EVs and drug-loaded liposomes. The plant-based EVs utilized, derived from *Hydrangea macrophylla* leaf (HML), were chosen for their potential synergistic anti-inflammatory efficacy [8]. Normal liposomes were fused with HML-EVs to confer additional anti-inflammatory properties, with the liposomes encapsulating terpinen-4-ol/azelamide monoethanolamine (TAM). Terpinen-4-ol, extracted from tea tree oil, exhibits remarkable antibacterial activity [9], while Azelamide MEA, an amide derivative of azelaic acid, possesses antibacterial and anti-inflammatory properties. Finally, the study aims to demonstrate the ability of the hybrid EV/liposomes to alleviate skin irritation induced by ultraviolet rays through *ex vivo* evaluation of reconstructed skin tissue.

Materials and Methods.

Fabrication of HML-EV

To fabricate HML-EV, HML was suspended in distilled water at a ratio of 1:20 (w/w). The mixture was then ground using a grinder. The resulting suspension underwent centrifugation at 3,000 × g for 20 minutes to eliminate impurities. The remaining residue was separated, and the supernatant was collected and centrifuged at 10,000 × g for 20 minutes. Further purification involved ultra-high-speed centrifugation at 150,000 × g for 2 hours, which resulted in the EVs

settling into the pellet layer. The EV-rich pellet was re-suspended in distilled water, filtered through a syringe filter with a 0.22 μm pore size, and stored at 4 °C.

Fabrication of TAM-liposome

Liposomes were synthesized using a high-pressure homogenization technique. Initially, polyglyceryl-10 stearate, hydrogenated lecithin, ceramide NP, and terpinen-4-ol were dispersed in glycerin. Concurrently, azelamide MEA, 1,2-hexanediol, and dipotassium glycyrrhizate were dissolved in distilled water. The oil phase was then integrated into the water phase using an agitator. The resulting suspension was subsequently extruded twice using a microfluidizer at 900 bar pressure.

Monitoring the Formation of Hybrid EV/Liposome

To monitor the formation of hybrid EV/liposome structures, fluorescence resonance energy transfer (FRET) was utilized with PKH26 and PKH67 dyes to label the membranes of TAM-liposomes and HML-EVs, respectively, according to the standard labeling protocol. The membrane fusion of PKH67-labeled HML-EVs and PKH26-labeled TAM-liposomes was initiated by mixing and proceeded during incubation. The fluorescence spectra changes were analyzed by mixing TAM liposomes and HML-EVs in a 1:1 (v/v) ratio. Fluorescence signals were measured with an excitation wavelength of 475 nm. The FRET efficiency was determined using the formula: FRET efficiency (%) = [(FA/(FD+FA))] × 100, where FA and FD represent the fluorescence intensities of the acceptor (PKH26) at 563 nm and the donor (PKH67) at 506 nm, respectively.

Nitric Oxide (NO) Assay

RAW 264.7 cells were seeded in 96-well plates at a density of 6×10^4 cells per well. After a 24-hour incubation, the culture medium was removed, and the cells were washed with PBS to

induce cell starvation. Subsequently, the cells were treated with varying concentrations of the samples in the presence of 1 µg/mL LPS. Following 24 hours of treatment, Griess reagent was added to an equal volume of the culture medium and incubated for 15 minutes at room temperature. The absorbance was measured at 560 nm. A standard curve for sodium nitrite was used to determine the optimal NO concentration.

TNF-α Assay

RAW 264.7 cells were cultured in 96-well plates at a density of 6×10^4 cells per well. The cells were then treated with various concentrations of the samples along with 5 µg/mL lipopolysaccharide (LPS). After 24 hours of treatment, the supernatant was collected, and the levels of tumor necrosis factor-alpha (TNF-α) were measured using ELISA kits. Absorbance was read at 450 nm using a microplate spectrophotometer. The concentration of TNF-α in pg/mL was determined by extrapolating from a standard curve generated using recombinant mouse TNF-α protein standard.

***Ex vivo* Assays of Inflammatory Responses**

In an *ex vivo* study of inflammatory responses, an oil-in-water (O/W) emulsion comprising 10 wt% hybrid HML-EV/liposomes was prepared using high-shear homogenization. The PAR-2 receptor was labeled with red fluorescence initially localized beneath the epidermis. Upon exposure to UV radiation (UVA 1 J/cm² and UVB 30 mJ/cm²), the red fluorescence spread throughout the upper epidermis. Post-UV stimulation, a noticeable reduction in red fluorescence was observed in the upper epidermis of the sample treatment group. Blue fluorescence denotes nuclear staining. The Thymic Stromal Lymphopoietin (TSLP) exhibited green fluorescence spanning the entire epidermal layer in the UV radiation stimulation control group, contrasting with the untreated control group. Interleukin-6 (IL-6) showed intense green fluorescence across the epidermal layer in the UV radiation stimulation control group compared

to the untreated control group, with additional green fluorescence observed in the cytoplasm of dermal layer fibroblasts. Following UV radiation stimulation, reduced green fluorescence was evident in both the epidermis and fibroblast cytoplasm of the sample treatment group.

Results.

The core achievement of our hybrid EV/liposome system was the successful induction of fusion between the vesicular membranes of TAM-liposomes and HML-EVs, thereby harnessing the intrinsic anti-inflammatory capabilities of each vesicular material. Mixing HML-EVs and TAM-liposomes initiated fusion between similar lipid bilayers, a process confirmed through TEM analysis (Fig. 1a-c). Both TAM-liposomes and HML-EVs exhibited typical vesicular morphology. In the dry state under high vacuum, the hybrid HML-EV/liposome displayed similar morphological features at a length scale of 100–200 nm. Notably, the periphery of the hybrid HML-EV/liposome exhibited a dark appearance akin to that of HLM-EV, suggesting the presence of EV membrane proteins, which were also observed in the traditional liposome, the TAM-liposome, possibly contributing to this resemblance [10]. The average hydrodynamic particle size of HML-EV and TAM-liposome was measured at 149.1 nm with a polydispersity index (PDI) of 0.27 and 151.1 nm with a PDI of 0.25, respectively. The hybrid HML-EV/liposome fell within the hydrodynamic particle size range of ~193.3 nm with a PDI of 0.19, exhibiting a zeta potential of ~-37 mV, which ensures adequate dispersion stability, akin to HML-EV (Fig. 2).

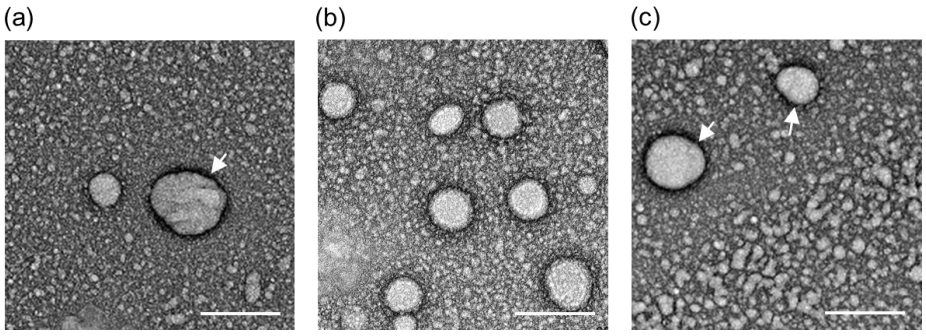

Figure 1. (a) TEM images of (a) TAM-liposome, (b) HML-EV, and (c) hybrid HML-EV/liposome.

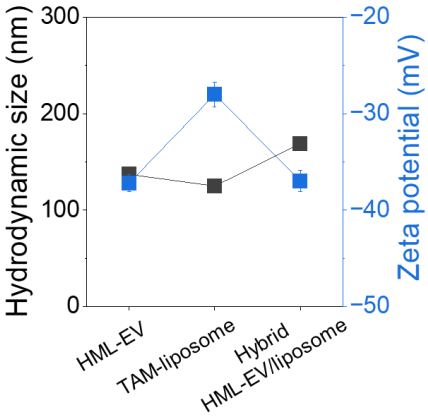

Figure 2. Hydrodynamic particle size and zeta potential of TAM-liposome, HML-EV, and hybrid HML-EV/liposome (n=3).

The hybrid EV/liposome was prepared by mixing TAM-liposomes with HML-EVs, followed by light vortexing and incubation at 4°C, with membrane fusion monitored using the FRET method. Successful hybrid formation was confirmed by FRET behavior utilizing the fluorescence properties of PKH67 and PKH26 [11] (Fig. 3a), showing FRET efficiencies after membrane fusion of PKH67-labeled HML-EV and PKH26-labeled liposomes were 0.12, 0.20, and 0.40 before mixing, and after 5 minutes and 1 hour of mixing, respectively. Flow cytometry showed colocalization of PKH67/PKH26 signals, indicating fusion (Fig. 3b). The highest fusion efficiency, 75%, was achieved with a 1:1 ratio of TAM-liposomes to HML-EVs. Optimizing the mixing ratio increased particle size, reflecting effective membrane fusion.

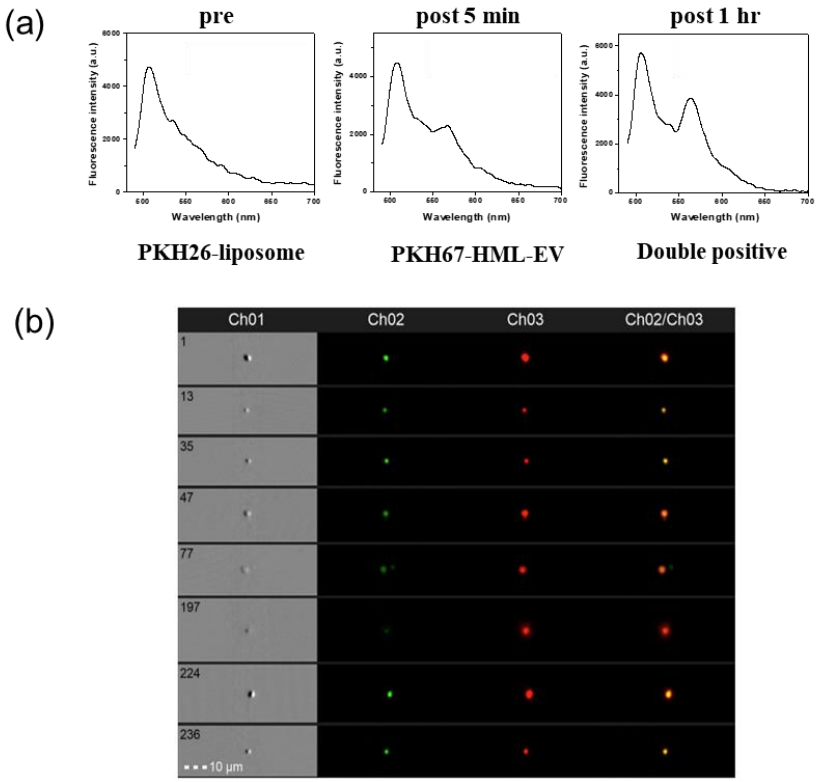

Figure 3. (a) Fluorescence spectra of PKH67-labeled HML-EV before mixing, and after 5 minutes and 1 hour of mixing with PKH26-labeled liposomes. (b) Single vesicle imaging using multispectral imaging flow cytometry.

The skin is directly exposed to UV rays that cause skin damage and aging [12]. UV exposure leads to excessive NO production in the skin, damaging lipids, proteins, nucleic acids, and enzymes, and accelerating inflammatory mediator production [13]. Therefore, anti-inflammatory ingredients are essential to alleviate UV-induced photodamage. We evaluated the anti-inflammatory effects of TAM-liposome, HML-EV, and hybrid HML-EV/liposome on LPS-induced RAW 264.7 murine macrophage cells. NO and TNF-α production were measured in LPS-activated RAW 264.7 cells treated with 0.5 to 5% (w/w) test samples, with no cytotoxicity observed at these concentrations. LPS stimulation significantly increased NO and TNF-α levels compared to the control group without LPS. Treatment with hybrid HML-EV/liposomes significantly reduced NO and TNF-α by approximately 5 pg/mL. As a result, the hybrid HML-

EV/liposome inhibited NO production by 39.1 ± 2.2% and TNF-α expression by 53.3 ± 1.9% (n = 5), outperforming the LPS-stimulated control, HML-EV, and TAM-liposome (Fig. 4a-b).

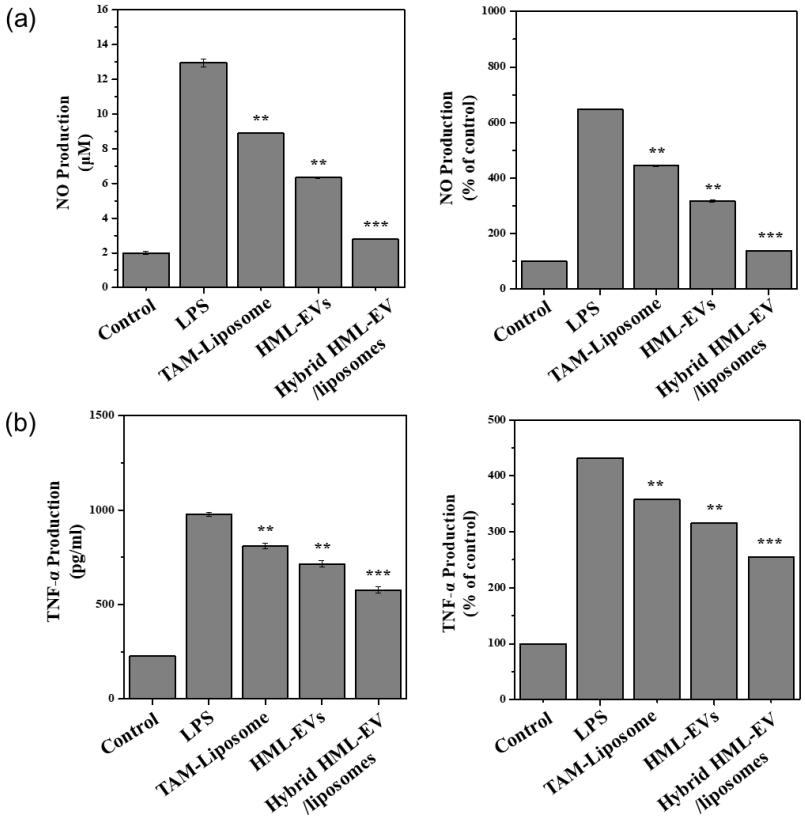

Figure 4. Effect of TAM-liposomes, HML-EVs, and hybrid HML-EV/liposomes on NO production and TNF-α expression in LPS-activated RAW 264.7 cells. (a) Effects of TAM-liposomes, HML-EVs, and hybrid HML-EV/liposomes on NO production in LPS-activated RAW 264.7 cells were measured by NO assay kit. (b) Effects of TAM-liposomes, HML-EVs, and hybrid HML-EV/liposomes on TNF-α expressions were determined by ELISA kits. The results are presented as mean ± standard deviation of triplicate data. (ns: *P<0.05, **P<0.01, ***P<0.001). Each bar represents the mean of three independent experiments.

After demonstrating the *in vitro* anti-inflammatory effects of the hybrid HML-EV/liposome system, we assessed its *ex vivo* skin anti-inflammatory activity against UV-induced PAR-2,

TSLP, and IL-6 overexpression. PAR-2, a G protein-coupled receptor implicated in inflammatory and metabolic disorders, regulates skin tumor formation and pigmentation in epidermal barrier keratinocytes [14]. UV-induced activation of PAR-2 triggers the release of inflammatory mediators such as IL-8, IL-13, TNF-α, and TSLP. TSLP, similar to IL-7, acts as an early stimulator of skin immune responses in keratinocytes, initiating the secretion of inflammatory cytokines and chemokines, and stimulating nerve cells associated with itching or scratching behavior [15]. To evaluate PAR-2, TSLP, and IL-6 expression in the skin, an oil-in-water (O/W) emulsion cream with 10 wt% hybrid HML-EV/liposome was applied to a reconstructed human skin (RHS) model [16]. Histological analysis of the sham group confirmed well-reconstructed RHS, consisting of the stratum corneum, epidermis, and dermis. UV radiation, penetrating the epidermal and dermal layers, induced skin irritation characterized by stratum corneum desquamation and swelling, along with a significant increase in PAR-2, TSLP, and IL-6 expression. Treatment with the hybrid HML-EV/liposome-containing emulsion reduced PAR-2, TSLP, and IL-6 production by 21.9%, 31.5%, and 32.2%, respectively, compared to the control group (Fig 5.). Moreover, the treatment also restored the thickness of the stratum corneum, similar to that of the sham group. These findings underscore the effectiveness of hybrid HML-EV/liposomes in inhibiting pro-inflammatory cytokines in UV-stimulated RHS models, alleviating skin irritation.

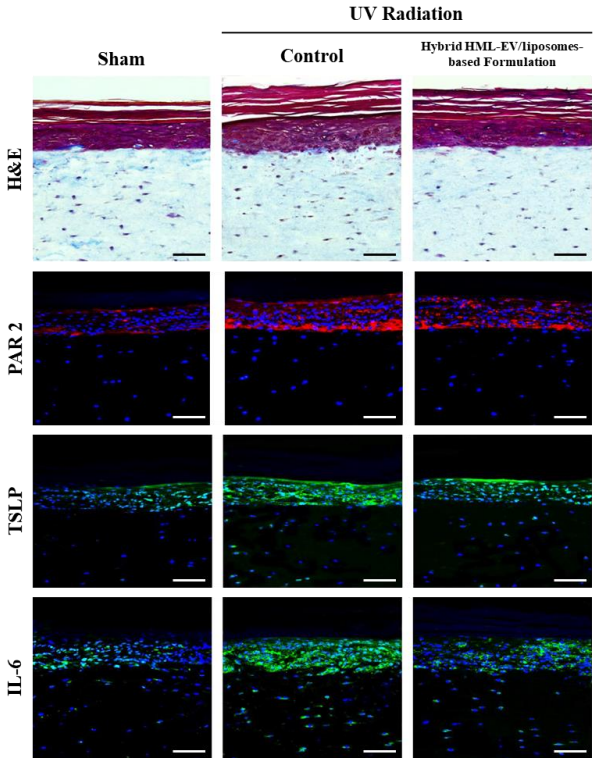

Figure 5. Histological anti-inflammatory effects of the hybrid HML-EV/TAM-liposome-based delivery system on the RHS. Representative microscopic images of H&E-stained RHS tissue sections, along with labeling for PAR-2, TSLP, and IL-6. Scale bars indicate 100 μm.

Discussion.

Our study demonstrates the successful creation of a hybrid EV/liposome system combining TAM-liposomes and HML-EVs, resulting in vesicular membrane fusion. These fusion harnesses the anti-inflammatory properties of both materials, confirmed through TEM analysis. The hybrid vesicles maintain similar morphology to parent vesicles and exhibit stable hydrodynamic size and zeta potential, crucial for anti-inflammatory therapies. FRET analysis confirmed the fusion, showing rapid kinetics and effective membrane integration. Flow cytometry indicated a high fusion efficiency of 75%, forming a single functional unit. The hybrid vesicles showed significant reductions in NO and TNF-α production in LPS-induced RAW 264.7 murine macrophages, suggesting superior anti-inflammatory efficacy due to synergistic

effects. These findings align with previous studies on the anti-inflammatory potential of liposomes and EVs. The fusion enhances these properties, offering a novel approach to inflammation treatment. The *ex vivo* skin model demonstrated practical applicability, reducing PAR-2, TSLP, and IL-6 expression in UV-damaged skin, indicating potential for topical formulations. The study's strength lies in the innovative combination of plant-derived EVs and liposomes, validated by multiple analytical techniques. However, *in vitro* and *ex vivo* models may not fully replicate human skin complexity, necessitating further *in vivo* studies for clinical confirmation.

Conclusion.

In summary, we have devised a practical technique for skin anti-inflammation by harnessing a hybrid HML-EV/liposome system, created through membrane fusion, to leverage the benefits of plant-based EVs and traditional liposomes. The successful formation of this hybrid HML-EV/liposome was confirmed through FRET measurements. Our research demonstrates that the hybrid HML-EV/liposome effectively inhibited NO and TNF-α production. Furthermore, the treatment with hybrid HML-EV/liposomes ameliorated skin inflammation induced by ultraviolet radiation by suppressing the expression of PAR-2, TSLP, and IL-6. These findings suggest that the hybrid HML-EV/liposome system developed in this study shows significant promise as a versatile platform for addressing skin inflammation in various dermatological and cosmetic applications.

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Conflict of Interest Statement.

NONE.

References.

1. Sriwidodo, A.K. Umar, N. Wathoni, J.H. Zothantluanga, S. Das, J.A. Luckanagul, Heliyon, 8 (2022) e08934.
2. C. Caddeo, O. Díez-Sales, R. Pons, C. Carbone, G. Ennas, G. Puglisi, A.M. Fadda, M. Manconi, J Colloid Interface Sci, 461 (2016) 69-78.
3. G. Fuhrmann, I.K. Herrmann, M.M. Stevens, Nano Today, 10 (2015) 397-409.
4. J. Quesenberry, J. Aliotta, M.C. Deregibus, G. Camussi, Stem Cell Research & Therapy, 6 (2015) 153.
5. H. De La Peña, J.A. Madrigal, S. Rusakiewicz, M. Bencsik, G.W. Cave, A. Selman, R.C. Rees, P.J. Travers, I.A. Dodi, J Immunol Methods, 344 (2009) 121-132.
6. Z. He, J. Wang, C. Zhu, J. Xu, P. Chen, X. Jiang, Y. Chen, J. Jiang, C. Sun, Cancer Lett, 548 (2022) 215751.
7 Y.T. Sato, K. Umezaki, S. Sawada, S.-a. Mukai, Y. Sasaki, N. Harada, H. Shiku, K. Akiyoshi, Scientific Reports, 6 (2016) 21933.
8. S. Yashaswini, R. Hegde, C. Venugopal, International Journal of Research in Ayurveda & Pharmacy, 2 (2011) 375-382.
9. L.M. Sun, C.L. Zhang, P. Li, J Agric Food Chem, 60 (2012) 6150-6156.
10. E.M. Guerreiro, B. Vestad, L.A. Steffensen, H.C.D. Aass, M. Saeed, R. Øvstebø, D.E. Costea, H.K. Galtung, T.M. Søland, PLoS One, 13 (2018) e0204276.
11. S. Aryal, T.D.T. Nguyen, A. Pitchaimani, T.B. Shrestha, D. Biller, D. Troyer, ACS Biomater Sci Eng, 3 (2017) 36-41.

12. A.T. Slominski, M.A. Zmijewski, C. Skobowiat, B. Zbytek, R.M. Slominski, J.D. Steketee, Adv Anat Embryol Cell Biol, 212 (2012) v, vii, 1-115.

13. M.Q. Man, J.S. Wakefield, T.M. Mauro, P.M. Elias, Inflammation, 45 (2022) 949-964.

14. M.K. Yau, J. Lim, L. Liu, D.P. Fairlie, Expert Opin Ther Pat, 26 (2016) 471-483.

15. Y. Zhang, B. Zhou, Immunol Res, 52 (2012) 211-223.

16. J.A. Bouwstra, R.W.J. Helder, A. El Ghalbzouri, Adv Drug Deliv Rev, 175 (2021) 113802.