Increased bio-stability and efficacy through surface modification of stem cell-derived nanovesicles drug carriers

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Ye Young Lee

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This certifies that the Master's Thesis
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Lastly, I hope that this thesis will be helpful to many people researching stem cell-derived therapeutics.

June 2024

Ye young Lee

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ABSTRACT

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Many studies have suggested that nanoparticles, such as liposomes and nanovesicles (NV), may be used as a therapeutic. However, there are challenges regarding in-vivo stability and particle stability. Therefore, PEGylation is emerging as a method for improving the stability of internal and nanoparticles. In order to be used as a therapeutic by applying the method, the PEG quantification shall be made before commercialization. Nanoparticles such as liposomes are made up of synthetic polymers, and they are easy to analyze using high performance liquid chromatography (HPLC) and gel permeation chromatography (GPC) as the compounds added to produce nanoparticles are well known. However, stem cell-derived nanoparticles consist of many compounds, such as protein and RNA, making it difficult to analyze their chemical properties due to the presence of complex recognition signals. However, information about PEG quantification is still insufficient. Thus, this study aims to investigate the methodology for PEG quantification per particle.

This study found that the high shear-rate dispersion method can produce PEGylation in large quantities using nanovesicle and suggested that PEG quantitative analysis method using produced PEGylated NV produced by the method. PEGylated NV produced by the method can be quantitatively and consistently controlled according to the displayed PEG concentration per particle. However, there are difference types of critical points that can be maximum displayed depending on the lipid-PEG type. Therefore, this study compared the difference between PEGylation critical points according to PEG based on two lipid types and the stability of particles according to the PEG density of the particle surface. DMG-PEG2000 (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000) displayed efficiency was about 20 times higher than DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-amino (polyethylene glycol)-2000), and the particle stability increased as PEGylation increased. This study also verified that

PEGylation could be used as a targeted therapeutic of NV through validation evaluations as it synthesis target material of the distal ends of PEG chains.

When a targeting peptide is displayed on PEGylated NV, target efficiency to lesions is increased; therefore, it can be used as a more effective drug delivery system. For example, therapy efficiency can be improved by increasing the mucosal permeability of the organ when using a PEGylated NV. DMG-PEG has higher PEGylation efficiency and hydrophilic than DSPE-PEG, and the advantages may vary depending on the purpose of the therapy. Therefore, further research is required to identify the chance of circulation time according to PEG density and patterns of cellular uptake depending on indications and to optimize the PEGylation density through in-vitro and in-vivo efficiency evaluation.

This study increases the biostability using PEGylation of human-friendly stem cell-derived NV and suggests a quantitative analysis method and critical point of PEGylation according to lipid-PEG types. Furthermore, we could confirm through validation that the medicinal effect of NV can be improved by displaying peptide on distal ends of PEG chains. Also, we could identify through the in-vivo that the therapeutic effect can be increased as PEG helps the adsorption of NV in the mucous membrane. These results indicate that cell-derived NV may be surface modification depending on the site and purpose of therapy, and quantitative analysis is possible.

Keywords: Extracellular vesicles, Nanovesicles, Stem Cells, Surface Modification, Biostability, PEGylation

I. INTRODUCTION

Extracellular vesicles (EV), commonly known as exosomes, are nano-sized vesicles that plays a role as an intracellular communicator. The EV size range is between 20~30 nm and consists of a lipid bilayer enclosing protein and nuclear. EVs get attention as a regenerative therapeutic as it affects the maintenance of homoeostasis, immune reaction, anti-inflammatory, and biological activating factors, including nucleic acid and protein in the vesicles. Also, EVs has a similar size and structure to lipid nanoparticles and liposomes. However, EVs is biocompatible, and they will have outstanding stability for blood circulates in the body as it has membrane protein [1-3]. The stability and validation should be maximized to develop an effective drug delivery system. Therefore, several studies have investigated the method to display targeted material in drug delivery systems such as liposomes, high-molecule nanoparticles and EV for optionally delivering and distributing drugs in targeted disease part as targeted therapeutic [4, 5].

Among the EVs for developing treatments, exosome derived from mesenchymal stem cells has been associated with improved heart attack, heart failure, ischemic disease and hepatic fibrosis [6]. Exosomes can combine stem cells biological advantages with nanoparticles structural advantages, making them effectively applicable for various indications and potentially very effective in disease treatment, as demonstrated by numerous studies. However, the exosome released by the cell is insufficient to commercialize, requiring high cost and time to separate, purify and concentrate high-purity exosome from the culture medium. Furthermore, poses difficulties in modifying a surface and external drug loading.

Pharmacokinetics control is essential to effectively deliver drug delivery systems based on nanoparticles such as exosomes and liposomes to targeted moiety. As far as it is concerned, the drug delivery system that circulates in the bloodstream is adsorbed by a mononuclear phagocyte system, leading removal of the drug delivery system. Avoiding capture by the phagocytic of the liver and spleen to extend circulation time is critical. It is known that the lipid composition, size, and zeta potential of nanoparticles affect circulation time[7-9]. Even though exosome show a significant

effect, the stability of particles and maximized treatment effect should be improved to be used as a treatment. Therefore, recent research has been conducted to supplement the challenges. There are several methods, such as adding substances in the phase of cell culture, ultrasonic wave and electroporation [10] however, it needs the subsequent process to insert a high molecule in the lipid bilayer by expanding the lipid bilayer structure using physical force. During this process, the stability of the particles may be compromised by external stimulus, and the effective factors loading in the cargo may leak out of the particle during the bilayer expansion. Also, it does not show a consistent effect, and significant particle loss may occur [6].

Therefore, producing nanovesicles, which are extracellular vesicles with a structure similar to exosome, by physically crushing and reassembling stem cells has been developed as an alternative method [11](figure 1). The production of NV is relatively easy for surface modification in the cell reassembly phase; therefore, the production process can be simplified, including modifying a surface. As a result, it may show consistent efficiency under the same conditions. Also, extracted NV amount is several hundred times higher than extracted exosome from the same amount of mesenchymal stem cells(MSC). NV have been reported to have validation similar to that of exosome in terms of pharmacological effects [12]. However, it is crucial to ensure the internal stability of nanoparticles, stability of the dispersion, ease of storage and distribution of the product to commercialize NV as a treatment [13]. Biopharmaceuticals made from biological materials are heavily influenced by temperature and environment and have limitations in maintaining physical and chemical stability, making it essential to ensure stability during storage, distribution, and re-dispersion in medical settings. Lyophilization is a method for easy storage, transportation, and long-term preservation for treatment that can be used without the expensive cold chain [14]. Many studies have been on preserving proteins that have a good effect or action after re-dispersing frozen or lyophilization EVs. Nevertheless, further studies are needed to increase particle stability [13, 15]. Particle surface modification through PEGylation can be considered to increase particle stability.

In the 1970s, Davis and Abuchowski found that covalent attachment of PEGylation to bovine serum albumin, and it has been used as a surface modification method in lipid nanoparticle(LNP), biodegradability nanoparticle and protein therapeutics[16]. PEGylation defines the modification of phosphatide, protein, drug or nanovesicle molecule by linking one or more polyethylene glycol

(PEG) chains that the FDA approves [17, 18] This polymer improves stability, circulation time and targeted delivery of molecules within the body[19].

PEGylation can be used through various methods, such as chemical combination or physical adsorption. PEGylation is a critical technology for improving in vivo stability by being applied in liposomes and lipid nanoparticles, as the PEG brush prevents aggregation of the particles. Therefore, it increases the effectiveness and circulating time by protecting molecules from transmutation. It has also been utilized in the COVID-19 vaccine.

PEGylation of NV during manufacturing enhances the re-dispersibility of particles by increasing interparticle repulsion and preventing aggregation [20]. PEGylated NV also has advantages in terms of biostability as it increases circulation time and, thus, is extended separate discharge. Therefore, it increases the probability of reaching disease sites. In order to extend the circulation time, cellular uptake be prevented. Several studies shown that protein adsorption can be controlled by surface PEG, which may verify the hypothesis that increases circulation time [21]. EV that are not applied PEGylation disappears from plasma within 5 minutes of injection, accumulates mostly in the liver and lungs within 10 minutes, and is separately discharged within 4 hours [22]. However, EVs that are applied PEGylation, accumulation in the liver is prevented by up to 30-50%[23]. Moreover, PEGylation is essential for NV to play as a targeted therapeutic. Because PEGylation can improve its role as targeted therapeutic and pharmacological effects as it is attached to targeting peptides, aptamers, proteins, and antibodies at distal ends of PEG chains[24, 25] [26]. There are a few things to consider for PEGylation: the complexity of synthesis varies depending on the type of lipid conjugated with PEG, making large-scale production challenging and more costly.

DMG-PEG is a lipid type attached to PEG chains by a glycerol linker. It is commonly used to modify the surface of liposomes or other nanoparticles to increase stability and circulation time in the body. In this study, we selected two PEG-conjugated lipids and compared their differences in terms of critical point and particle stability of PEGylated NV. It is DSPE-PEG and DMG-PEG. There are a few differences between DSPE-PEG and DMG-PEG. The main difference between DSPE-PEG and DMG-PEG is how the PEG chain is attached to the lipid molecule. DSPE-PEG is attached via amid bond, whereas DMG-PEG is attached via glycerol linker. Another difference between

DMG-PEG and DSPE-PEG is the size of the chain that is attached to lipids. DSPE- DSPE-PEG typically has a longer PEG chain than DMG-PEG, resulting in a relatively long half-life. Also, it is insoluble in water and has a lower critical micelle concentration (CMC). Therefore, it has lower PEGylation efficiency. DMG-PEG has a shorter half-life but is relatively soluble in water and has a higher CMC, resulting in higher PEGylation efficiency (Figure 2). The difference in lipid attached to PEG can affect the mobility and characteristics such as stability in vivo, circulation time and targeted delivery. Also, there is a limitation regarding the size. When pegylated, the particle size increases, making it difficult to infiltrate specific tissue and cell, and it is not easy to apply to the cell-derived vesicle. In the case of liposomes, PEGylated occurs during the synthesis process to make particles; therefore, it is easy to be PEGylated and control the amount. However, cell-derived vesicles have formed a phospholipid membrane. Inserting PEG into the phospholipid membrane is challenging as pegylated occurs in the subsequent process, and even more challenging to control the amount. Additionally, cell-derived vesicles contain numerous components other than PEG, making it difficult to analyze PEG quantitatively.

Various methods for analyzing PEGylation have been reported, such as labelling PEG with fluorescence, confirming ascending sizes with PEG, and confirming the surface charge. However, these methods are indirectly evaluated, and quantitative analysis is complicated[27, 28]. Among drug delivery systems, liposomes extensively studied for surface modification have been widely used with PEGylation [21, 29-32]. A various approach using NMR, proton, HPLC and GPC for determination of liposomes have been used as liposomes are empty in inside of particle and composed of high purity PEG compared to other substances. However, NV and exosomes derived from cells contain numerous factors such as lipids, proteins, nucleic acids, and various metabolites on their surface, which may overlap with the PEG signal and make it difficult to distinguish. Also, it is difficult to completely dissolve the particles in a deuteride solvent, making it difficult to analyze them quantitatively using methods such as liposomes due to the uncertainty of pretreatment for determination[24, 33]. Therefore, this study suggests the effectiveness and expectation of the quantitative analysis methods of PEGylated PEG and PEGylation that can produce large-scale nanovesicles during simultaneous PEGylation.

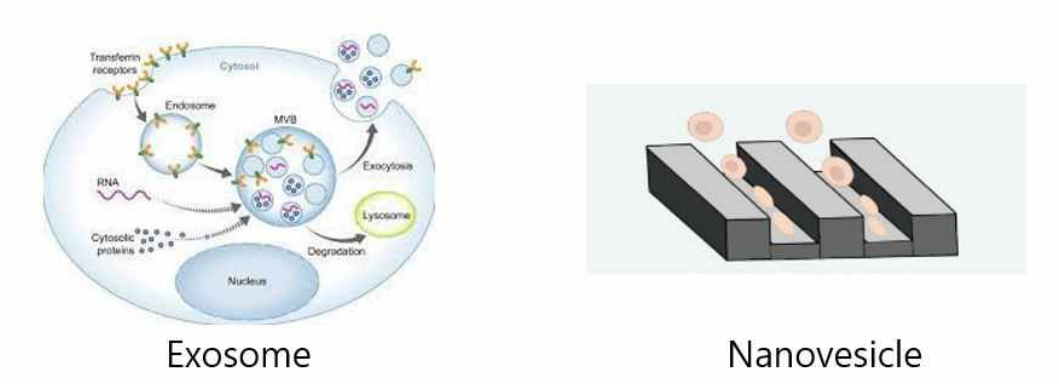
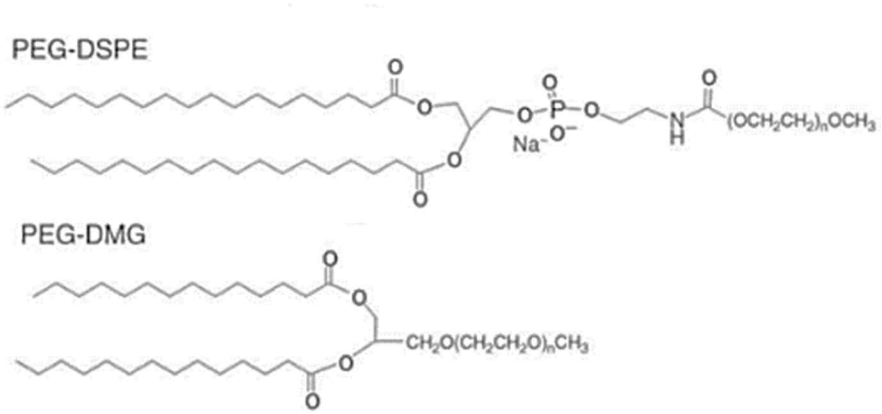


Figure 1. Difference between exosome and nanovesicle

The exosome is an extracellular vesicle that is released by cells. Nanovesicles is the particle that physically crushes the cell. During the cells high shear-rate fluid dispersion process, neighbor substances can be adsorbed or involved.

A. PEG-lipid molecular



B. Comparison of differences in hydrophilicity according to types of PEG-conjugated lipids

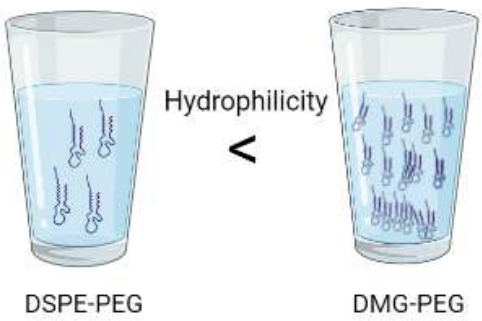


Figure 2. PEG-lipid molecular structure

This is the molecular structure of PEG-DSPE and PEG-DMG. DMG lipid tail is 14, and DSPE is 18. Therefore, DMG is shorter than DSPE, which implies DMG is hydrophilic and critical micelle concentration may vary. B. The reason why PEGylation can be better is that it is more soluble in buffer.

II. MATERIALS AND METHODS

1 Cell culture

This study used adipose-derived mesenchymal stem cells (ADMSC) isolated from the fatty tissue of 20's females. The culture fluid of cells (Gibco, Thermo Fisher, USA) was cultured with DMEM supplemented with 5% penicillin and 10% human platelet lysate(hPL). It was seeded at 5,000 cell/cm^2 density and incubated at 37℃ with 5% CO_2 for five days. The cells were trypsinized with 0.05-0.25% trypsin-EDTA(ethylene-diamine-tetra acetic acid) for 5 minutes and neutralized with a solution containing 1% human platelet lysate to make a suspension that included cells. The cells were centrifuged at 900g for 10 min to obtain the cell pellets.

Human umbilical vascular endothelial cells (HUVEC) used in in-vitro experiments were cultured using Endothelial Cell Growth Kit supplemented with an Endothelial cell basal medium (Promocell, Germany). HUVEC was sub-cultured at a seeding density of 10,000 cells/cm2 and grown to confluency in 150 cm^2 cell culture flasks. It was cultured for five days at 37℃ with a 5% CO_2 incubator. The culture medium was replaced every 2-3 days. Glass bottom culture chambers (SPL, Korea) were used for cell imaging, and 12-well plates (SPL, Korea) were used for qPCR. All cells were cultured at 37℃ with 5% CO_2, and the culture medium was replaced every 2-3 days.

2 Nanovesicle isolation and PEGylation

The ADMSCs in pellet form were diluted in PBS to a concentration of 2E+6/mL and mixed with Polyethylene glycol (PEG) at a specific concentration to produce a solution. DMG-PEG2000(Avanti, USA) and DSPE-PEG2000(NOF, Japan) were used as PEG. The cell suspension that redispersed the stem cell was passed through a microchannel with a diameter of 75μm at a pressure of 5000 psi using a high-pressure homogenizer. In this process, the stem cells were distributed and crushed by a high shear rate that occurs in the microchannel and it formed stem cell-derived nanovesicles by reassembling in the microchannel. The shear rate generated in the microchannel was calculated to be 4.4E+6 to 5.9E+6. The lysis cell by high shear-rate fluid dispersion method became PEGylation as self-assembly (Figure 3).

The reassembled stem cell-derived nanovesicles were suspended in PBS buffer and then purified using the tangential flow filtration(TFF) method. The size-exclusion purification and concentration were performed using TFF(KR2i, Repligen, USA). The solution was filtered through a 750kDa filter using the solution diluted in PBS as 0.1% F68 to remove small proteins, debris, and residual surfactants that did not participate in PEGylation.

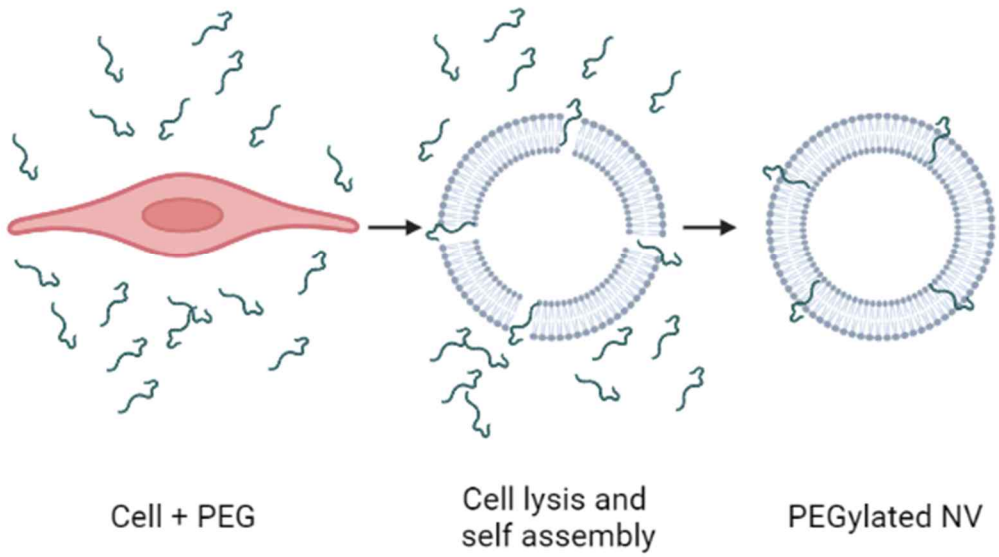


Figure 3. NV preparation and PEGylation process

The cells become lysis, and PEG molecules are inserted in bilayer lipids during self-assembly.

3 Characterization

3.1 Nanoparticle Tracking Analysis (NTA)

The concentration and average size of particles produced by Nanoparticle Tracking analysis were measured to investigate the impact of two PEGylated methods on the stability of cell-derived particles. Nano Sight NS3000(Malvern, United Kingdom) was used for the measurement. The samples were diluted 10,000-fold in PBS, and 1mL was loaded for each measurement, carried out for 40 seconds, four times, to obtain the average value.

3.2 Cryo-Transmission electron microscopy (cryo-TEM)

The size and particle size of the particles have been verified by analyzing the core support center at Seoul National University Cell and Giant Macral Imaging. The analysis was conducted using a 200kV Jeol 2100P, and the specimen production was loaded from the RT using the Quantifoil Grid (1.2/1.3) with Thermo Vitrobot.

3.3 Surface marker analysis of NV

CD63, CD81, and CD9 antibody are coated with exclusive exosome chips (Nanoview Biosciences, USA) to Incubated overnight at RT in the dark, and rinsed 3 times. Optionally, an antibody with fluorescent NV was labeled and confirmed by the Nanoview biosciences (USA).

3.4 NV stability

Non-PEGylated NV, DMG-PEG2000 5μM, 80μM concentration is added to diluted with PBS in line with the particle concentration of 1e+11/mL. Store at 4℃. for 9 to 11 days under static conditions. Digital Rotator (RK-1D, DAIHAN, Korea) exposes the shear stress condition for 88

hours in room temperature (RT) at 20 rpm speed. The stability of the NV particles is checked in Optical Microscope (Olympus, JAPAN) using capillary tube.

4 Cell viability

HUVEC were seeded on 96 well plate at a density of 1E+4 cells/well and were allowed to adhere for overnight. This study investigated cell proliferation of Non-PEGylated NV and PEGylated NV using CCK-8 assay to confirm whether PEGylation disturbed NV effects and endotheliocyte of NV protection effect. The PEGylated NV group was prepared by adding DMG-PEG2000 at 10μM and 100μM each and DSPE-PEG2000 at 10μM for the experiment. The group was divided into three as follows: Positive control group (none treat), TNF-α control group (TNF-α 20ng/mL with PBS) and Dysfunction group (TNF-α 20ng/mL with NV 1E+11particles/mL). They were treated for 12, 24, and 72 hours. The CCK-8 assay solution (dilution 10%, Dojindo, Japan) was added and incubated for 4 hours at 37℃ with 5% CO_2, and the absorbance was measured at 450nm using a multi-plate reader (Victor Nivo, PerkinElmer, USA).

5 Cytotoxicity and anti-inflammatory effect

Anti-inflammatory activity was evaluated in-vitro in a cell-based model of inflammation using 100 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich). For compared non-PEGylated NV with PEGylated NV, Positive control group (none treat), LPS control group (LPS 100ng/mL with saline) and Dysfunction group (LPS 100ng/mL with NV 1E+11particles/mL) was determined. Raw 264.7 cell were seeded on 12 well plate at a density of 1E+5 cells/mL and were allowed adhere for 2 days. After treatment with PEGylated NV (DMG-PEG2000 0, 5, 20 or 50μM) for 24 hours, cells were pelleted by centrifugation. Total mRNA was extracted from the cell pellet using the RNA isolation kit (Thermo Fisher, USA), and cDNA was generated using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher, USA). TNF-α, IL-1β and IL-6 mRNA levels were analyzed by real-time polymerase chain reaction (RT-PCR). The RT-PCR conditions were as follows: 10 minutes at 95℃ for the initial denaturation, followed by 50 cycles of 15 seconds at 95℃, 30 seconds at 58℃, and 30 seconds at 72℃. A melting curve analysis was performed following amplification. mRNA levels were determined according to the $^{\Delta\Delta}$CT method.

6 Method for quantification of PEGylated PEG and peptide conjugation

6.1 HPLC-ELSD

PEG and non-PEGylated NV were analyzed by HPLC-ELSD (1290 Infinity 2, Agilent, USA). Column uses Poroshell CS-C18 2.7μM, 2.1 x 100mm, Mobile phase A uses 5mM Ammonium formate in DW : CAN : MeOH = 33.3 : 16.6 : 50, B uses 5mM Ammonium formate in MeOH : EtOH = 60 : 40 and the flow rate was measured under the conditions of 0.6mL/min, column temperature at 40 °C, and gas flow rate at 1.6 SLM.

6.2 1H NMR

Non-PEGylated NV and PEGylated NV were measured at 600 MHz 1H NMR (JNM-ECZ660R, Jeol, Japan). $CDCl_3$ was used as the solvent and pyridine 100 μM was used as the standard material. The sample used lyophilized NV and the measurement was scanned 64 times. To obtain quantitative results, the T1 relaxation time (T 1) of PEG and pyridine was measured, and the accuracy was improved by setting the delay time (D 1) to 5 times the PEG T 1 [34]. Total PEG quantification by NMR completely dissolved lyophilized PEGylated NV in $CDCl_3$ [35].

6.3 mPEG ELISA

Sandwich mPEG ELISA (Methoxy-Peg Elisa Kit, Life Diagnostics, USA) was used. The concentration of NV was loaded with 1E+8/mL ~ 1E+6/mL (n = 3) and the standard curve was obtained using the DSPE-PEG2000 used in the PEGylation as a standard material. As an experimental group, buffer, non-PEGylated NV, and PEGylated NV were set. The concentration of the DSPE-PEG2000, which was added during the manufacture of NV, was composed of 1, 4μM.

6.4 Peptide conjugation

DSPE-PEG2000-NHS (NOF, JAPAN) and sequence are mixed with SDKP peptide (Genscript, USA) with molar ratio 1: 2, 1: 4. The solvent synthesizes in RT using N, N-Dimethylformamide (DMF). N_2 purging dialysis for 1 day in DW and replace DW every 6H.

7 In-vitro

7.1 Protein adsorption

NV that was produced by adding DMG-PEG2000 40 at 80μM concentration, Non-PEGylated NV and BSA (Bovine serum albumin, VWR Chemicals, USA) were prepared.

NV particles were set as a 1E+12/mL, BSA 1mg/mL concentration, and diluted as PBS. After incubating for 20 minutes at 37℃, 100kDa dialysis bags (Repligen, USA) were used to remove the BSA that was not attached to NV for two days. PBS was used as a buffer and changed every 12 hours. When Dialysis was finished, the NV particle concentrations of each sample were adjusted to 8E+11/mL, and absorbance was measured using a BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, USA).

7.2 Cellular uptake and Immunofluorescence staining

1 mL of NV was prepared at a concentration of 1E+12/mL, and DiO or DiD (diluted to 1uL per 1mL of buffer, Vybarant, Invitrogen, USA) was incubated in the dark at RT for 30 minutes for labelling. Residual dye in labelled NV was removed using a PD-10 column with Sephadex G-25 (Cytiva, USA).

HUVEC were cultured, and a dysfunctional environment was induced by shaking the cells on a digital rocker in a culture medium for 24 hours. After 20 hours, 1E+12 particles of labelled NV were added and incubated for 4 hours. After removing the NV-containing culture medium, the cells were washed twice with DPBS. NV 1E+12 particles were treated for staining and incubated for 4 hours. After 24 hours, the culture medium mixed with NV was removed and washed twice with DPBS. The NV treated cells were fixed with 4% araformaldehyde for 15 min at RT. Cells were permeabilized with 0.1% Triton X-100 in TBST for 10 min at RT, followed by washing 2 times with TBST. 5% BSA was treated for 30 min at RT to block the nonspecific binding. The cells were incubated with a VCAM-1 primary antibody (dilution 1 : 250, Abcam, UK) for 2h at RT. The cells

were rinsed at least 2 times with TBST and treated a Goat anti-rabbit IgG Alexa Fluor® 488 secondary antibody(dilution 1:1000, Abcam, UK) and DAPI (1 drop/mL, NucBlue™ Fixed Cell Ready Probes, Invitrogen, USA), F-actin probe(1 drop/mL, Actin Red 555, Invitrogen, USA) for 2h at RT in the dark, then rinsed two washed with TBST. Fluorescence imaged were acquired using a fluorescence microscopy (Evos M5000, Thermo Fisher, USA) and Thunder imaging system (Leica, Germany).

7.3 Endothelial protective effect of NV

Endothelial protective effect was evaluated in vitro in a cell-based model using 5 ng/mL TNF-α. Furthermore, SDKP peptide conjugated PEG was used to confirm the PEGylation effect. The Positive control group(none treat), TNF-α control group(TNF-α 5ng/mL with saline)and ysfunction group (TNF-α 5ng /mL with NV 1E+11particles/mL) was determined. HUVEC were seeded on 12 well plate(SPL, Korea) at a density of 1E+5 cells/mL and were allowed adhere for 2 days. After treatment with PEGylated NV (DSPE-PEG-peptide 0, 10 μM) for 24 hours, cells were pelleted by centrifugation. Total mRNA was extracted from the cell pellet using the RNA isolation kit (Thermo Fisher, USA), and cDNA was generated using the High-Capacity RNA to cDNA™ Kit (Thermo Fisher, USA). VCAM-1, IL-6 and IL-8 mRNA levels were analyzed by real-time polymerase chain reaction (RT-PCR). The RT-PCR conditions were as follows 10 minutes at 95°C for the initial denaturation, followed by 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C. A melting curve analysis was performed following amplification. mRNA levels were determined according to the $^{\Delta\Delta}$CT method.

8. Statistical analysis

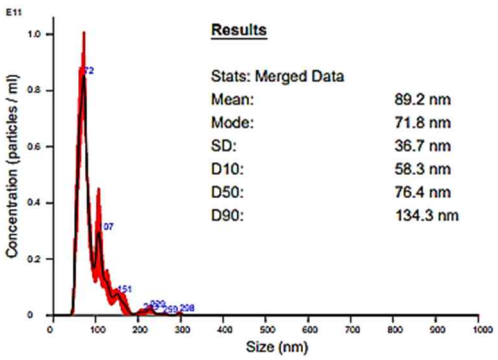
Prism 8 software was used to statically significant. The significance level was set at 0.05. ONE-WAY ANOVA was performed to investigate the levels between the control and experimental groups.

III. RESULT

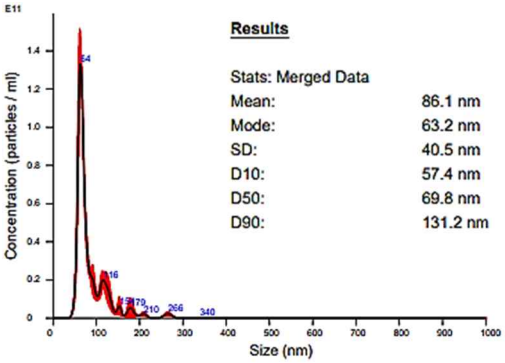
1 PEGylated NV Characterization and purification

The diameter of the pegylated-non-PEGylated NV, which was manufactured by high shear-rate dispersion and then purified, had a mean diameter of 86.1nm and a mode of 63.2nm. In comparison, the diameter of PEGylated NV had a mean diameter of 89.2nm and a mode of 71.8nm, indicating that PEGylation increased the size of the NV. Cryo-TEM confirmed that the particles were spherical. It was confirmed that the particles had CD63, CD81, and CD9, which are significant proteins on the surface of extracellular vesicles using fluorescent-labelled antibodies (Figure 4). To confirm that all non-participating PEG was removed during purification, non-PEGylated NV and PEG solution was mixed and purified using the same method. The feed and permeate were measured by 1H NMR. Pyridine 100mM was added to the solvent as a standard substance for normalization. The peak was not observed at 3.65ppm in the sample purified by adding non-PEGylated NV and PEG but was observed in the PEGylated NV. There was a significant difference in peak intensity before and after purification (Figure 5). It was confirmed that PEGylation of NV using self-assembly is possible and that removal of non-participating PEG is possible using the TFF system.

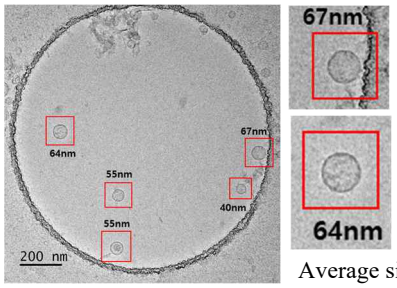
A. Particle size analysis: Bare NV



B. Particle size analysis: PEGylated NV



C. Particle size and shape confirmation (Cryo-TEM) : Bare NV



Average size : 63nm, STD : 28nm

D. Genetic marker identification: Bare NV

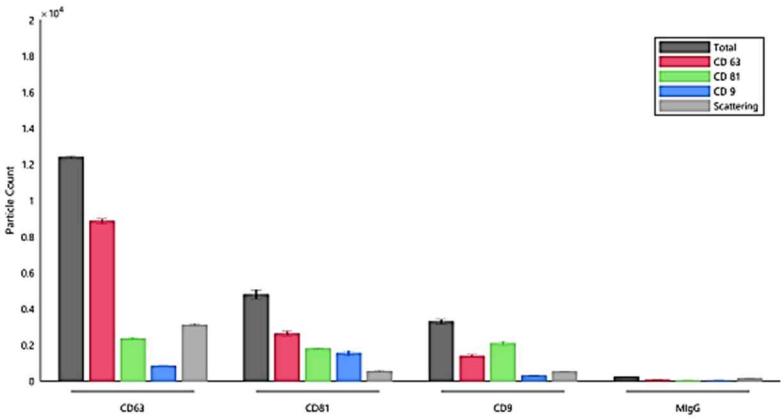
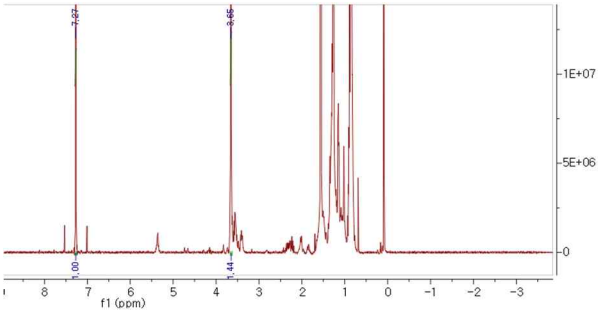


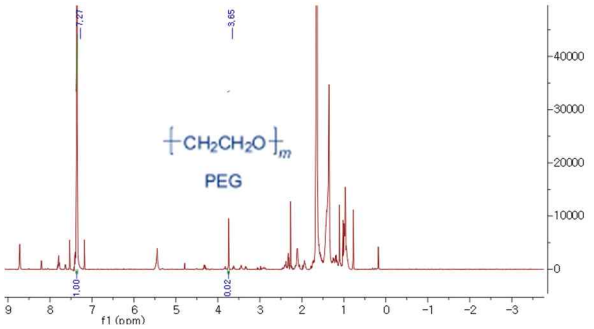
Figure 4. Characterization

Graph A shows the particle size distribution of NV by Nanoparticle Tracking analysis. They are Bare NV and B. PEGylated NV. The average size was 86~89nm. The form of NV was spherical, determined by TEM, and the particle size was 40~67nm. There was a genetic label that confirmed the stem cell-derived extracellular vesicles.

A. Before PEGylated NV by TFF filtration (NMR spectrum)



B. Non-PEGylated NV + PEG by TFF filtration (NMR spectrum)



C. After PEGylated NV by TFF filtration (NMR spectrum)

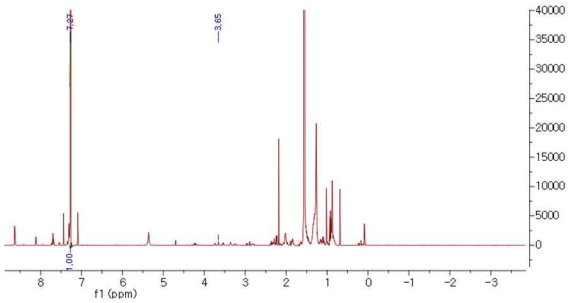


Figure 5. Remove PEG that did not participate in PEGylation through the tangential flow filtration system

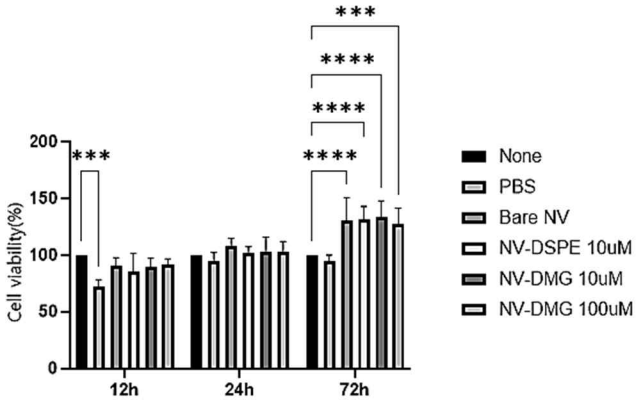
A filter removes PEGylation that is not involved in the production of PEGylated NV. A. Prior to TFF filtration, NMR spectra were obtained. B. After TFF filtration of non-PEGylated NV and NV+PEG, the NMR spectra were normalized using the solvent peak at 7.27 ppm, and the PEG peak at 3.65 ppm was compared. The results indicate that PEG molecules not involved in PEGylation were removed during the TFF process. C. After TFF filtration of PEGylated NV, the NMR spectra confirmed that PEG molecules involved in PEGylation were preserved and not removed during the TFF process.

2 Confluency and cytotoxicity

Cell proliferation and anti-inflammatory efficiency in HUVEC were confirmed using CCK-8 assay and qPCR. Cell proliferation showed a statistically significant increase in treated samples after 72 hours, while non-treated samples that only buffered(PBS) showed a similar result. When non-PEGylated NV and PEGylated NV were compared, there was no statistically significant difference (Figure 6-A). Also, the impact of the amount of PEGylation in qPCR was investigated. As a result, when three different NV groups were compared, including non-PEGylated NV and PEGylated NV at varying concentrations, TNF-a decreased or remained at a similar level. In contrast, the pro-inflammatory cytokine IL-1β decreased as the PEGylation concentration increased. The cytokine IL-6, involved in inflammation, metabolism, and regeneration, showed an increasing trend with PEGylation concentration, but there was no statistically significant difference compared to non-PEGylated NV (Figure 6-B).

Therefore, it was concluded that NV has no toxicity in HUVEC and has an anti-inflammatory effect based on the experiment results. Also, the hypothesis could be made that cellular uptake happens with increasing PEGylation of DMG-PEG.

A. EC(HUVEC) proliferation



B. Anti-inflammatory effect

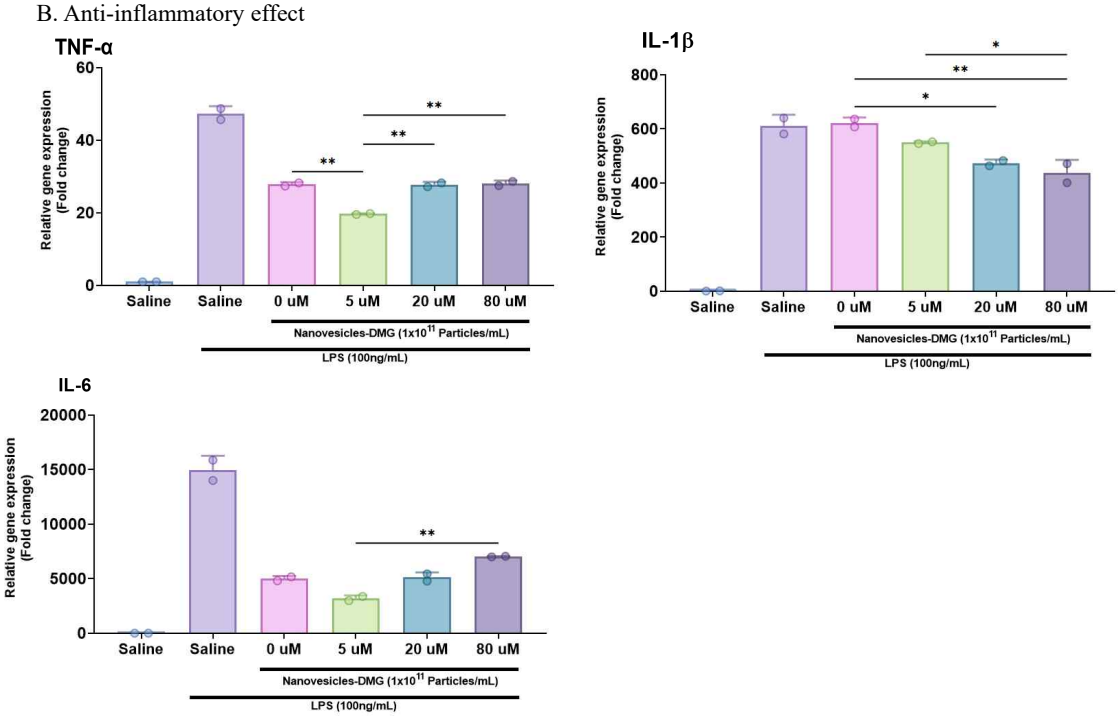


Figure 6. Proliferation and anti-inflammatory effect

A. Bare and PEGylated NV proliferation increased when it was investigated in HUVEC using CCK-8 assay (Compared with negative control group) B. The anti-inflammatory effect was analyzed by qPCR according to PEGylation concentration. NV and PEG are non-toxic, and the stem cell-derived extracellular vesicle efficiency on anti-inflammatory was confirmed. *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

3 Selection of analysis method for PEGylation

3.1 HPLC-ELSD

Non-PEGylated NV and DSPE-PEG (2000) were measured in order to detectability. As a result, it was concluded that quantitative analysis was impossible because the substance was detected simultaneously as passing through the column (Figure 7-A).

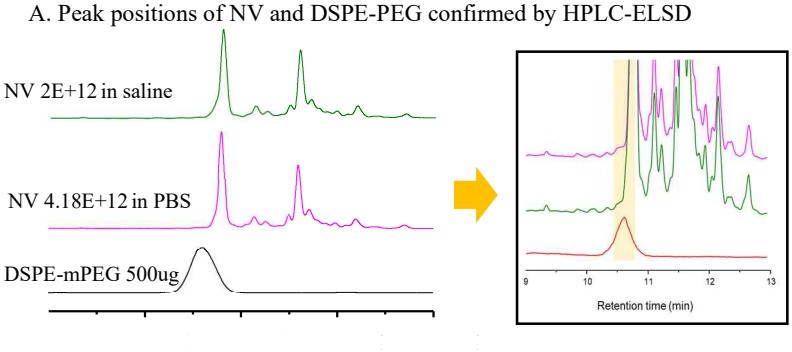
3.2 mPEG ELISA

Quantification was attempted according to the PEGylation concentration with elisa of the mPEG sandwich method. When manufacturing NV, it was measured to be an average of 1000 per particle at a DSPE-PEG (2000) addition concentration of 4μM and an average of 500 at 10μM. It may be seen that there is a critical point between 4μM and 10μM in the addition concentration of DSPE-PEG (2000) (Figure 7-B).

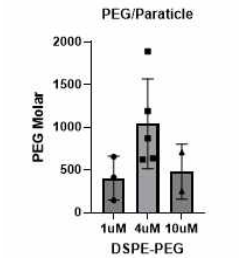
3.3 1H NMR

To confirm detectability, non-PEGylated NV and PEGylated NV were compared. The most suitable quantitative method was used because there was an overlapping peak at 3.65 ppm to the presence of PEG. Additionally, pyridine (8.62 ppm, 7.15 ppm, 7.69 ppm) was checked as a standard substance to confirm that its peak did not overlap with NV and PEGs (Figure 7-C).

A. Peak positions of NV and DSPE-PEG confirmed by HPLC-ELSD



B. PEG quantification of PEGylated NVs identified by mPEG ELISA



C. Bare NV and PEGylated NV confirmed by 1H NMR

[Bare NV] [NV-DSPE-mPEG 4μM]

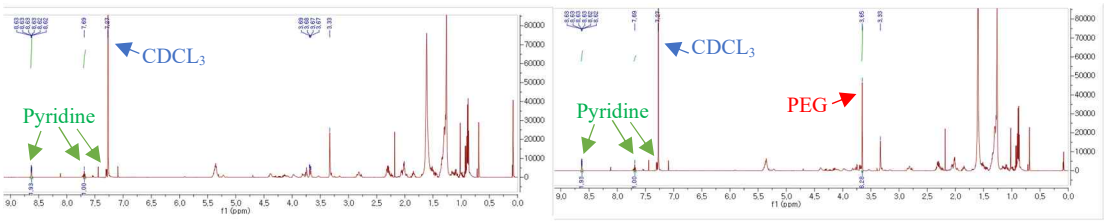


Figure 7. PEGylation analysis method

PEG that becomes PEGylation quantity analysis . A. According to the analysis by HPLC-ELSD, many substances appear similar to DSPE-mPEG in the region necessary for quantification. Although the actual DSPE-mPEG is also slightly visible, it is difficult to quantify accurately. B. It was confirmed by mPEG ELISA. (1μM : n=3, 4μM : n=5, 10μM : n=2) Although the sandwich ELISA method was used for confirmation, it is difficult to find evidence that all particles are bound to anti-PEG, and the range of error is extensive. All graphs are plotted with means +/- standard deviations. During the 1H NMR analysis, it was confirmed that the peaks of NV and PEG do not overlap. It was confirmed to be an appropriate analysis method as it does not overlap with the peak of pyridine, which was selected as the standard substance.

4 PEGylated PEG concentration analysis and optimization

4.1 1H NMR

The result of T1 relaxation time shows that pyridine, selected as a standard substance, was 6.556 seconds, and PEG was 1.253 seconds. A delay time of 35 seconds, five times longer than pyridine, was set, and a 600MHz 1H NMR experiment was conducted. The method to calculate the efficiency of PEGylation based on the results of the analysis is as follows: The integration of the pyridine peak at 7.68-7.7 ppm was normalized, and the integration of the PEG peak (3.65 ppm) was obtained. The H proton of each substance was determined, and the concentration of PEG was determined relative to the standard substance. The concentration was multiplied by Avogadro's number to determine the number of PEG molecules. The number of PEG molecules involved in display per NV particle can be obtained by substituting the number of sampled NV when analyzing NMR.

$$\frac{Pyridine\ \ Integration\ \ \div 1}{PEG\ \ Integration\ \ \div (PEG\ \ H\ \div Pyridine\ \ H)} = PEGylated\ \ PEG\ (\mu M)$$

$$(6.02 \times 10^{23}) \times PEGylated\ \ PEG\ (M) = PEGylated\ \ PEG\ (molecule\ \ )$$

$$\frac{PEGylated\ \ PEG\ (molecule\ \ )}{NV\ particles\ \ of\ \ NMR\ sample} = PEGylated\ \ PEG/particle$$

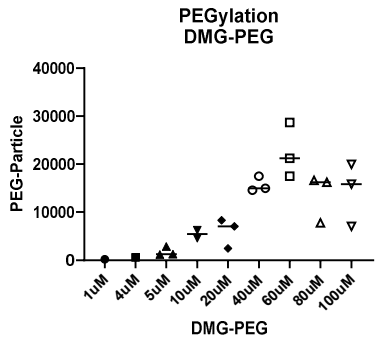
DMG-PEG showed a critical point at an additive concentration between 60-80μM. PEGylated PEG molecules are about 17,481 per particle (Figure 8-A). DSPE-PEG showed a critical point at an additive concentration of 5μM. PEGylated PEGs are about 760 per particle (Figure 8-B, Table 1).

4.2 ζ potential

When comparing the ζ potentials according to PEGylation presence, non-PEGylated NV showed a potential of -30.1, NV-DSPE showed a potential of -18.3, and NV-DMG showed a potential of -13.1. The surface charge of the particles became neutral due to the PEG. (Figure 8-E).

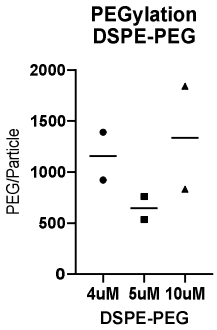
A. NV-DMG-PEG PEGylation

Quantitative Data Confirmed by NMR

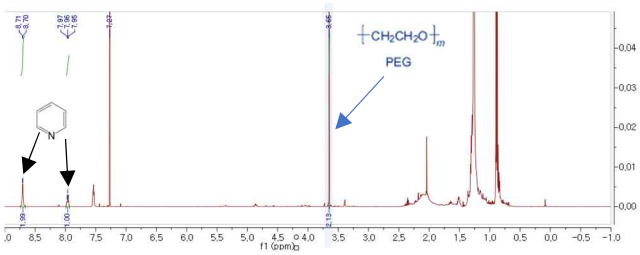


B. NV-DSPE-PEG PEGylation

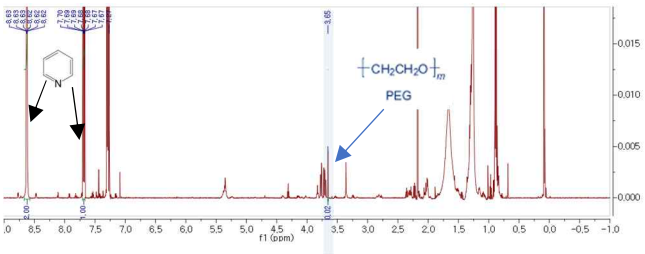
Quantitative Data Confirmed by NMR



C. NV-DMG-PEG 100μM (NMR spectrum)



D. NV-DSPE-PEG 5μM (NMR spectrum)



E. Comparison of negative charge

Figure 8. 1H NMR : Determination of PEGylation quantity and critical point

A and B are PEGylation quantification data for DMG-PEG and DSPE-PEG. As standards, pyridine can be confirmed at 7.68 ppm and PEG at 3.65 ppm. They represent the number of PEG molecules involved per NV particle. The critical point of DMG-PEG was observed at additive concentration 60~80μM, and their PEG/Particle was about 17,481. The critical point of DSPG-PEG was observed at additive concentration 5μM, and their PEG/Particle was about 760. C, D: 1H NMR 600 MHz spectra. E is a graph comparing zeta potential according to PEGylation presence. The bare NV is -30.1, NV-DSPE is -18.3, and NV-DMG is -13.1, indicating that the surface charge of the particles became neutral due to the PEGylation.

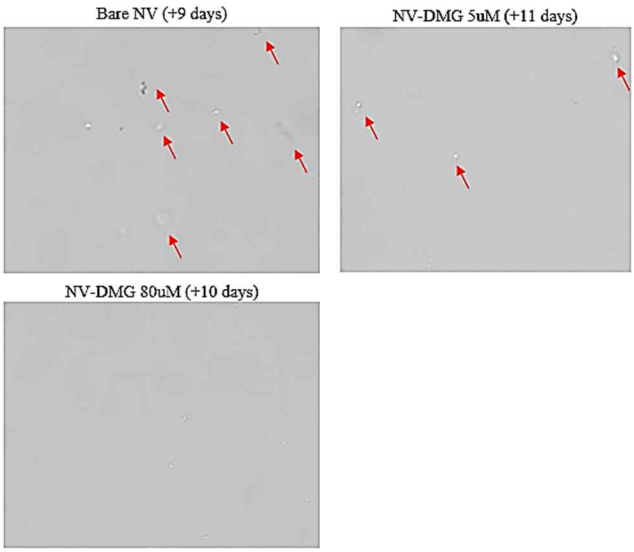
Table 1. Quantification of PEGylation by NMR result

Lipid	Concentration of PEG added (μM)	PEGylated PEG (mg)	Number of PEGylated Molecules	Total particles	PEG/ particle	area/PEG (nm^2)	Efficiency of PEGylation (%)
DMG	1	0.3	1.8E+14	4.66E+13	181	173.6	14
DMG	4	1	6.0E+14	4.43E+13	603	52.1	9
DMG	5	2	1.2E+15	2.50E+13	1206	26.0	10.4
DMG	5	2.1	1.3E+15	4.26E+13	1266	24.8	23.2
DMG	5	4.8	2.9E+15	4.17E+13	2893	10.9	50.1
DMG	10	7.6	4.6E+15	5.14E+13	4581	6.9	24.4
DMG	20	4.1	2.5E+15	2.20E+13	2471	12.7	4.4
DMG	20	13.8	8.3E+15	4.42E+13	8319	3.8	34.2
DMG	20	11.7	7.1E+15	4.61E+13	7053	4.5	30.5
DMG	40	24.2	1.5E+16	4.15E+13	14588	2.2	16.9
DMG	60	29	1.7E+16	4.14E+13	17481	1.8	11.9
DMG	80	12.9	7.8E+15	2.88E+13	7776	4.0	4.6
DMG	100	11.7	7.1E+15	4.97E+13	7053	4.5	5.8
DSPE	4	0.32	1.4E+15	5.02E+13	1390	22.6	26.7
DSPE	4	0.122	9.2E+14	5.01E+13	922	34.0	10.2
DSPE	5	0.076	5.4E+14	3.15E+13	536	58.5	5.1
DSPE	5	0.135	7.6E+14	3.74E+13	760	41.3	9.0
DSPE	10	0.46	1.8E+15	5.34E+13	1842	17.0	15.3
DSPE	10	0.094	5.7E+14	3.57E+13	567	55.4	3
DSPE	10	0.111	8.3E+14	4.48E+13	832	37.7	3.7

5 NV particle stability

The stability of particles according to the concentration and PEGylation presence was indirectly observed in static and shear stress conditions using an optical microscope. It was observed that the particle agglomeration of PEGylated NV was less than that of non-PEGylated NV in both conditions. A high concentration of PEGylation showed less agglomeration in static conditions (Figure 9). Therefore, we could confirm through this experiment that PEGylation affect the stability of particles.

A. Comparison of particle agglomeration between bare NV and PEGylated NV under shear stress conditions



B. Comparison of particle agglomeration between bare NV and PEGylated NV under static conditions

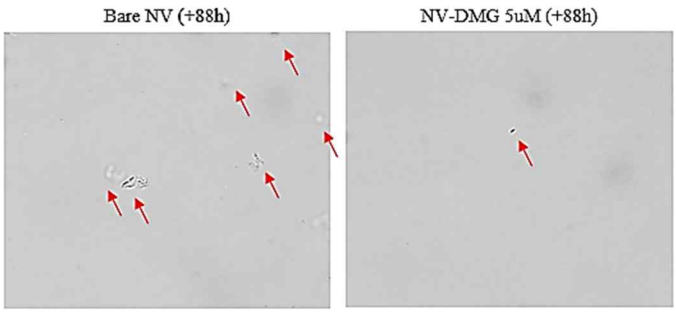


Figure 9. Particle Stability of PEGylated NV

The stability of the particle was investigated by optical microscope according to PEGylation presence and concentration in static and B: shear stress conditions. Agglomerate was less in the samples that had higher PEGylation.

6 The influence of PEGylation concentration on protein adsorption

The experiment was conducted to identify protein adsorption and determine how much it affects circulation. When NV is intravascularly injected, cell adhesion can proceed rapidly. In this case, the circulation time in the body is shortened, so the efficiency of reaching the lesion site is reduced. As an in-vitro experiment for this, a protein adsorption experiment was conducted (Figure10). It was evaluated based on the adsorption of BSA according to PEGylation. The amount of protein was calculated by assay. Compared before and after BSA adsorption, non-PEGylated NV increased by about 9.5 times, NV-DMG 40μM by 2.7 times, and NV-DMG 80μM by 2.9 times (Table 2). This result confirmed that PEGylation could prevent the adsorption of protein and can extend the time of circulation in the body.

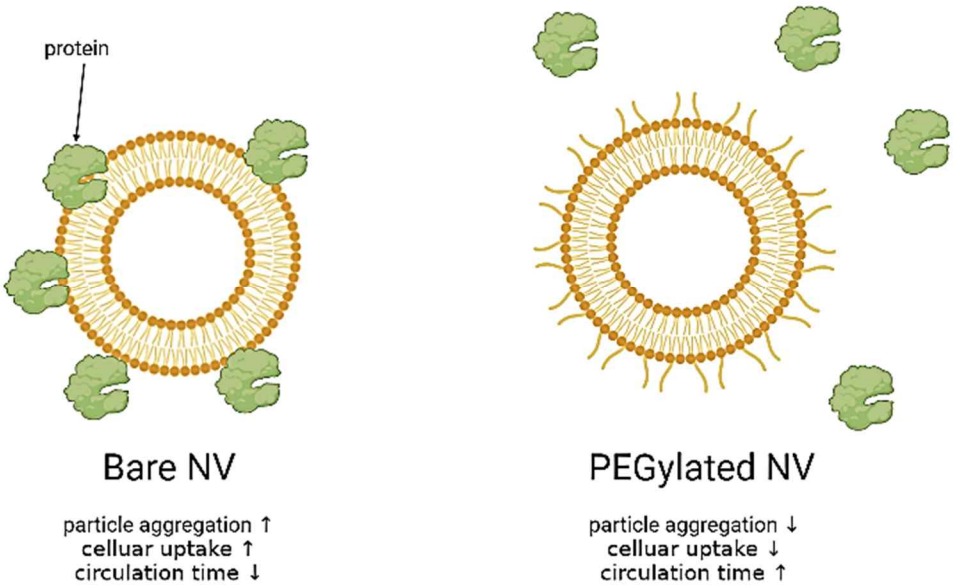


Figure 10. Differences and effects of protein and NV adsorption in the body

Table 2. BSA adsorption

Sample	Before BSA adsorption(ug/mL)	After BSA adsorption(ug/mL)	Incremental Multiplier
Bare NV	18	171	9.5
NV-DMG 40uM	49	135	2.7
NV-DMG 80uM	38	112	2.9

*8E+11 particles/mL

7 Intracellular uptake and location of NV

7.1 Dye removal

Fluorescence-labeled was performed to confirm the cellular uptake of NV in HUVEC. Fluorescence not labelled in NV needs to be removed to get accurate results. When the dye was diluted in buffer and passed through a PD-10 column with G-25 resin, the fluorescence value decreased by 2.75 times, similar to the buffer level without added fluorescence (Table 3). The particle showed a 20% loss; therefore, it showed the usable yield (Table 3). Therefore, based on the results, the PD-10 column may be suitable for dye removal in the solution that includes NV.

7.2 Peptide conjugation

CPP was labelled at the distal end of the PEG to distinguish the PEGylation presence. Molar ratio 1:4 showed the highest efficiency as it was 96% when DSPE-PEG-NHS and peptide were conjugations and analyzed by HPLC-ELSE and UV (Figure11, Table 4).

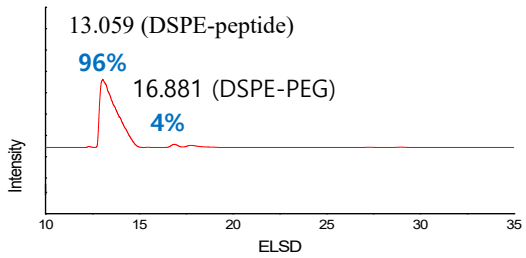
Table 3. Residual dye filtration

This study investigated whether the unreacted dye was removed from NV after DiO staining by dispersing it in PBS. After passing through a PD-10 column, the absorbance level was similar to that of PBS, confirming that all dye was adsorbed and removed. Additionally, the particle yield of NV was determined to be approximately 20% loss.

	Fluorescent value avg. (n=3)	Particles/mL (Origen 1E+12)
Before PD-10 column	3838401	7.76E+11
After PD-10 column	1394881	6.26E+11
PBS	1210852	-

*The fluorescence value decreased by 2.75 times after using the column. (Similar with PBS)

A. PEG-peptide conjugation confirmed by HPLC-ELSD



B. PEG-peptide conjugation confirmed by HPLC-UV

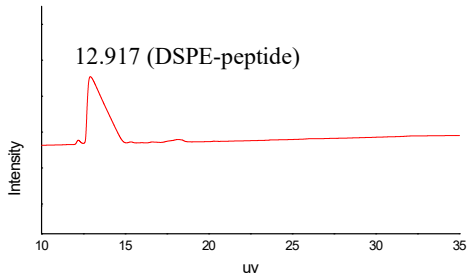


Figure 11. Peptide conjugation analysis

HPLC analyzed the efficacy after the conjugation of DSPE-PEG-NHS and peptide. A. HPLC-ELSD, B. Analyzing the graph of HPLC-UV. DSPE-PEG-NHS : Ac-SDKP = 1 : 4 (mol ratio) synthesize (DMF was used as a solvent, Overnight, RT, N_2 purging, Dialysis in DW)

Table 4. Peptide conjugation yield by ELSD

Reaction ratio (DSPE-PEG-NHS:peptide)	**TEA added**	**DSPE-peptide**	**DSPE-PEG**
1:2	0	65%	34%
1:4	Peptide molar x 5	96%	4%
1:4	0	91%	9%

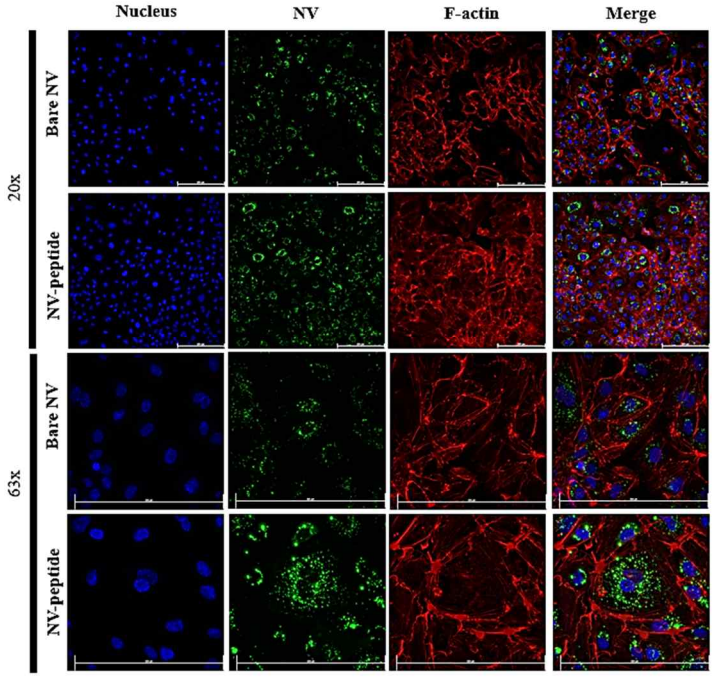
7.3 HUVEC chemically, mechanically dysfunction induction and validation of NV

When a high-resolution fluorescence microscope was used to determine the difference between non-PEGylated NV(Bare NV) and peptide labelled NV (NV-peptide) after inducing chemically dysfunction in HUVEC, it showed that NV peptide was more uptake than non-PEGylated NV (Figure 12-A). It was observed that more cells were present in the treated NV-peptide sample when counting the nucleus in the images (Figure 12-C). When the NV uptake area was normalized to the nucleus for analysis, NV-peptide was found in a greater area and showed statistically significant differences (Figure 12-D).

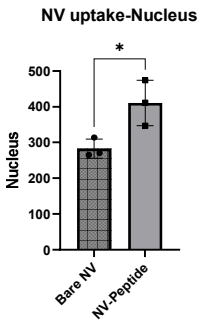
When mechanically dysfunctional HUVEC cells were induced, it was confirmed that sufficient VCAM1 expression occurred, and an in-vitro model of vascular disease was formed (Figure 13-A). When non-PEGylated NV and NV-peptide were compared to the experiment group, NV-peptide disturbed more VCAM1 induction. Statistically, significant differences were observed (Figure 13-B). All image analysis was performed using Image J (n=3).

When assessing the protective effects of HUVEC cells using qPCR, all experimental groups treated with NV showed efficacy compared to the non-treatment group. NV-peptide showed statistically significant differences in all selected indicators of validation. In contrast, non-PEGylated NV showed significant differences in IL-8 and VCAM1 (Figure 13-C).

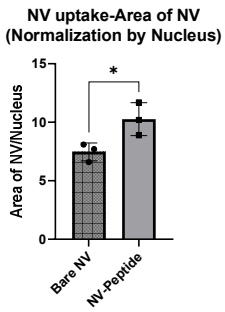
A. Comparison of cellular uptake between bare NV and peptide conjugated NV



B. Cell Survival



C. NV uptake areas



D. NV uptake areas per each EC

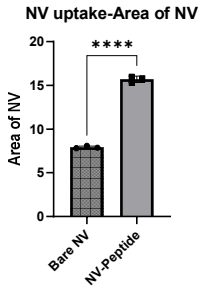
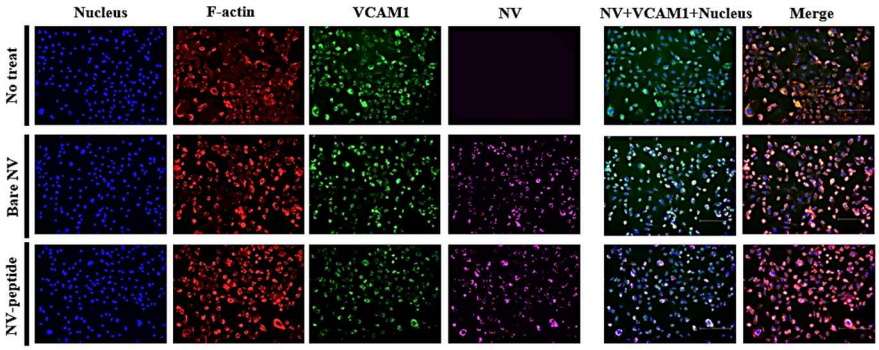


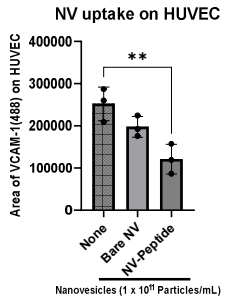
Figure 12. Cellular uptake

DiO stained NV was treated with HUVEC, and uptake of NV was confirmed after 4 hours. A. Fluorescence microscopy image. When comparing NV uptake displaying bare NV and PEG-peptide, it was confirmed that NV displaying PEG-peptide was taken up more. B. Graph counting the nucleus in the image, C. Graph measuring the NV uptake area. D. Graph shows the quantifies of the NV uptake area normalized by the nucleus. All image analyses were performed using Image J. (All graphs are plotted with means +/− standard deviations. Compared with Bare NV: *p<0.05, ****p<0.0001)

A. NV and VCAM1 colocalization



B. NV uptake by EC(HUVEC)



C. Endothelial protective Effect of Nanovesicles

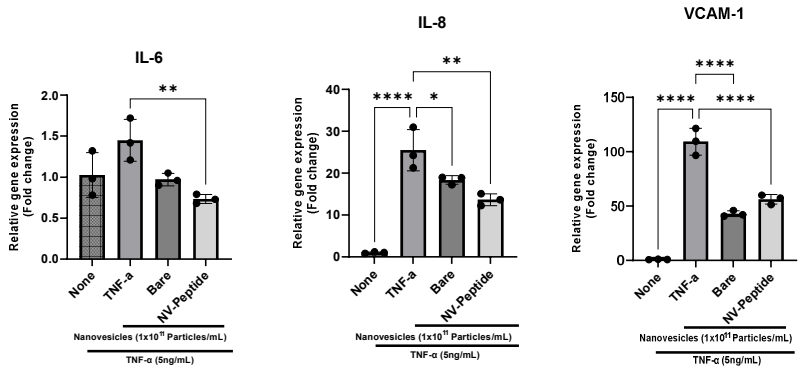


Figure 13. Confirmation of efficacy of vascular endothelial cell protection

A. Co-localization between NV and VCAM1 expression was confirmed by fluorescence microscopy. B. Graph analyzed from fluorescence microscopy images using Image J and normalized to the nucleus. (ONE-WAY ANOVA ** P<0.01) C. Graph showing the protective effect of the vascular endothelial cell at the gene level confirmed by qPCR. (Graphs are plotted with means +/− standard deviations. Compared with TNF-α: *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

IV. DISCUSSION

NV has limited effectiveness as it cannot target the specific cell or tissue of the body. Also, the effectiveness can be reduced as it has low stability; therefore, it is easy to decompose or agglutinate in the body. Also, it is difficult to be commercialized as a therapeutic due to the difficulty of production. Consistent and large-scale production of NV may be complex. The steric hindrance effect is one of the key benefits of PEGylation, as it helps to prevent the non-specific binding of nanoparticles to unwanted molecules and cells. It is necessary to adjust according to the purpose considering the effect of steric hindrance. A high PEG density can create a physical barrier that reduces the accessibility of functional groups on the nanoparticle surface. This can limit the ability of the nanoparticle to interact with target molecules or cells, potentially reducing its therapeutic efficacy. Therefore, PEGylation need to carefully optimize the PEG density to balance the steric hindrance effect against the need for sufficient surface functionality. Another potential is that the steric hindrance effect can reduce the cellular uptake of nanoparticles. While the steric barrier can help to prevent the adsorption of serum proteins by the immune system, it can also reduce the interaction of the nanoparticle with cell surface receptors. This effect can be particularly problematic in some drug delivery applications where high cellular uptake is necessary. It should be considered when using targeting peptide that located at the end of PEG. The steric hindrance effect can also affect the stability of nanoparticle formulations. High PEG densities can increase the steric repulsion between nanoparticles, making them more resistant to agglomeration. While the steric hindrance effect is a key benefit of PEGylation. Therefore, this study developed a method to prevent NV agglomeration, increase stability, and enable large-scale production. It was found that this method could maintain or even improve the efficacy of NV and could potentially show better efficacy.

1 Necessity and quantitative analysis of PEGylation

PEGylation improves stability and circulation time and can be a targeted therapeutic for specific cells or tissues. PEG density affects efficacy. The average distance (D) of PEG neighbors the Nanovesicle surface affects the structural form of PEG and PEGylation efficacy. The form of polymer can be explained by Flory radius (R*F*). The chain of PEG does not overlap and forms a mushroom when *the R F* chain is longer than D [36].

$R_F = aN^{3/5}$

(α= Å monomer dimension; α in case of PEG = 3.5 Å, N=Degree of Polymerization)

Increasing the density of PEGylation (R F/ D > 1) requires nearest neighbors PEG chains to overlap, and PEG must take on a brush structure[37]. It is generally known that protein adsorption is reduced when PEG has a brush-like shape, and it can avoid absorption by MPS cells [36, 38]. Another study found that nanoparticles PEGylated with 3.4kDa PEG at a density of R F/ D > 2.5 showed a 90% reduction in protein adsorption in human plasma compared to those that were not PEGylated[39]. Increasing the amount of PEG on lipid NV led to a ζ potential close to neutral, indicating that the negatively charged NV surface was more heavily PEGylated with PEG [40]. However, a method for determining PEG density based on surface charge has not been reported. Similarly, while a significant increase in circulation time was observed for PEGylated lipid nanoparticles, increasing the amount of PEG in the formulation by a factor of two did not increase circulation time[5]. These results suggest that increasing the PEG content during nanoparticle manufacturing may not necessarily increase the density of PEGylated PEG. Additionally, there may be a critical threshold for the maximum achievable PEG surface density that can vary depending on the type and manufacturing method of the nanoparticle. Therefore, accurately quantifying surface PEG density is essential when interpreting the influence of PEG density on nanoparticle circulation.

There are three methods were used for this study: ELISA, HPLC-ELSD and 1H NMR. When performing the experiment using from sandwich method for PEGylation quantity, loading particles in well should be binding to capture antibody. In this study, the NV sample was loaded with a new capture antibody to confirm the presence of captured particles during the second loading. The results showed that captured particles were not observed during the second loading when the particle concentration was below 1E+7/mL. However, it cannot be claimed that all particles were captured during the first loading (Table 9). An appropriate amount of particles must be used to confirm whether loading particles in the wall-bound to capture the antibody. This method is costly and has a significant data variation. HPLC-ELSD is faster and easier to analyze than other methods if the initial analysis conditions are set according to the solvent and substance. However, quantitative analysis is impossible for NV, which has many internal factors, because the lipid-PEG used for PEGylation overlaps with the peak. It is suitable for analyzing substances with simple and precisely known properties, such as liposomes or LNP. However, it is not suitable for analyzing cell-derived particles.

1H NMR was the most suitable method to analyze the quantity of PEG in PEGylated. The NV used in this study did not overlap with the PEG peak, and a solvent that can be used as a standard was found. The necessary pre-treatment conditions for this analysis are to remove the PEG that did not participate in PEGylation and find a solvent in which the freeze-dried NV is soluble.

Among the three analyzing methods, 1H NMR was the most suitable based on the results of this study. Therefore, further research requires to investigate cross-validation analysis.

Table 5. mPEG ELISA

Sample	Dilution	Initial measurement	Re-measurement after sample collection	Re-easurement -buffer	Residual particles
Buffer		0.099~0.146	-	-	-
NV-DSPE 1uM (particle : 1E+8/ml)	10000x	1.518	0.59	0.444	38 %
NV-DSPE 4uM (particle : 1E+8/ml)	10000x	2.388	1.171	1.025	49 %
NV-DSPE 4uM (particle : 1E+7/ml)	100000x	0.395	0.2395	0.0935	slightly
NV-DSPE 4uM (particle : 1E+6/ml)	1000000x	0.202	0.1165	-0.0295	-
NV-DSPE 1uM (particle : 8E+7/ml)	100000x	0.514	0.162	0.034	slightly
NV-DSPE 4uM (particle : 8E+7/ml)	100000x	0.972	0.266	0.138	31 %
NV-DSPE 4uM (particle : 7E+6/ml)	1000000x	0.178	0.117	0.02	slightly

2 PEGylation threshold and difference between DSPE-PEG and DMG-PEG

The critical point of PEGylation can vary depending on the bonding of lipids properties. Dissolving PEG with a high concentration in the solvent is critical for the efficacy of PEGylation from already formed particles such as extracellular vesicles. In particular, particles composed of proteins must exist in a water-based buffer rather than an organic solvent. The PEG solution that must be mixed during the process should be dissolved in a similar buffer type. Depending on the type of conjugated lipid, the CMC (critical micelle concentration) can vary, with DMG being more soluble in water at a higher concentration than DSPE, resulting in higher PEGylation efficiency. However, lipid-PEG should not be chosen solely based on high PEGylation efficiency but on the properties suitable for the intended purpose. In protein adsorption experiments, it was indirectly confirmed that the group with a higher density of DMG-PEG had a longer in vivo circulation time with less protein adsorption. This result indicates that the in vivo circulation time can be prolonged depending on the concentration of PEG. When indirectly confirming particle stability using an optical microscope, less aggregation was observed as the density of PEG increased. However, although the target peptide used in this study could be synthesized with PEG using amine, it was impossible to label the target peptide with DMG-PEG as NHS could not be synthesized. In addition, DMG-PEG with high PEGylation efficiency has a shorter half-life than DSPE-PEG, so it can be eliminated relatively quickly when injected into the body. This should be considered when administering the particle intravascularly. Considering the increase in membrane permeability and particle stability in proportion to the PEGylation concentration, DMG-PEG may be effective for intramuscular injection and mucosal administration.

3 Cellular uptake of NV labeled with CPP

The cellular uptake was performed by displaying Cell-Penetrating Peptide (CPP) in the distal ends of PEG to identify the possibility of targeting, which is the best thing for PEGylation and efficacy. Non-PEGylated NV and CPP-DSPE-NV(NV-peptide) that first production was used to the difference.

This study used fluorescence microscopy, so labelling NV with fluorescence and removing all residual dye is vital. G-25 resin may effectively remove the residual dye, but this process also has about 20% particle loss. However, it is superior to dialysis regarding particle stability, time, and dye removal efficiency.

NV uptake into HUVEC was analyzed by image and normalizing to the nucleus, NV-peptide was statistically significantly observed, and co-localization with VCAM1 was also confirmed. However, the size of NV is petite as a typical microscope cannot detect it, but the fluorescence displayed in NV can be detected. This means it is difficult to distinguish whether it is on the surface of HUVEC or has been absorbed into the cell by endocytosis and has reached the nucleus and whether what is seen under the fluorescence microscope is NV or only the fluorescent label remains. Therefore, the results of this experiment indicate that PEGylation of NV with synthesized PEG containing CPP helped cellular uptake and NV co-localized with VCAM1.

Therefore, further research is needed to analyze the time it takes for NV to reach the target cell and its degradation.

4 HUVEC protective effect of NV and cytotoxicity

There was no toxicity when the assessment of the efficacy and toxicity of NV, but it shows the efficacy of anti-inflammatory and protection effects to vascular endothelial cells. Firstly, the harmful effect of PEG was investigated. In the cell proliferation assay, both the NV treatment group and the non-treatment group increased, and there was no statistically significant difference between non-PEGylated NV and PEGylated NV. Furthermore, we confirmed whether the amount of PEGylation affects the anti-inflammatory effect using qPCR. As a result, when comparing with different densities of PEG, there was no significant difference in IL-1β compared to non-PEGylated NV. We confirmed that NV has anti-inflammatory effects and protects endothelial cell survival and that PEG does not inhibit or harm NV effects.

The vascular endothelial cell protection effect at the genetic level was compared regarding CPP display in the distal end of PEG to determine the peptide labelling by deepening the confirmation of cell proliferation. NV-peptide tended to have greater efficacy in IL-6 and IL-8 than non-labelled NV, and there was no statistically significant difference in VCAM1.

Although all experimental groups treated with NV showed some effect, it was not a dramatic difference. In in vitro experiments, since cells are treated with a high concentration of NV in an enclosed environment, it is easy for a large amount of NV to be uptake making it challenging to confirm the effect of targeted peptides. Despite this, the fact that there was a difference suggests the findings are promising, and further in vivo studies are necessary.

V. CONCLUSION

Therefore, the conclusions are as follows: PEGylated nanovesicles can be produced by mixing cell suspensions and PEG, followed by high shear-rate dispersion that causes cell lysis and self-assembly. This process allows for successful large-scale production and PEGylation simultaneously, with PEG molecules inserted during self-assembly. PEGylation increases the particle size by approximately 10nm and a change in particle surface charge to neutral. Quantitative analysis of PEGylation can be achieved through 1H NMR by calculating the proton H value of PEG compared to standard substances. DMG-PEG has a higher PEGylation critical point than DSPE-PEG, and particle stability increases when observed under a microscope.

The toxicity of NV and PEG in HUVEC was not observed; however, it showed the protection effect. The efficiency was increased when the labelled peptide was in the distal end of the PEG. Cellular uptake can be observed more than non-PEGylated NV during the same time.

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ABSTRACT (IN KOREAN)

줄기세포 유래 나노베지클 약물전달체의 표면개질을 통한 생체안정성 및 효능 증대

리포좀, 나노베지클을 비롯한 나노파티클은 입자 안정성, 체내 안정성을 향상시키기 위한 방법으로 페길화가 제시되었고 활발한 연구가 이루어지고 있다. 이 방법을 적용하여 치료제로 상용화하기 위해서는 전시된 PEG 의 양을 정량화 하여야 한다. 합성고분자로 이뤄진 리포좀과 같은 나노파티클은 제조 시 첨가된 합성물을 정확하게 알고 있어 HPLC, GPC 와 같은 분석을 적용하기 쉬우나 줄기세포 유래 나노파티클은 단백질, RNA 등으로 입자를 구성하고 있는 성분이 많아 화학적 분석 시 복잡한 시그널이 확인되어 같은 방법을 적용하기 어렵다. 그렇기 때문에 줄기세포 유래 나노파티클을 페길화하여 연구한 기존 문헌에서는 입자 당 전시된 PEG 를 정량화 한 내용을 찾기 힘드나 본 연구에서는 이를 정량화 할 수 있는 분석 방법을 개발하였다.

본 연구에서는 나노베지클을 페길화하면서 대량 생산할 수 있는 방법인 고전단율 유체분산 방법을 고안하고 해당 방법을 통해 제조된 페길화 나노베지클의 페길화 된 PEG 의 정량적 분석법을 제안하였다. 개발한 방법으로 제조된 페길화 나노베지클은 입자 당 전시된 PEG 를 농도에 따라 정량적, 일관적으로 조절할 수 있다. 하지만 PEG 에 접합된 지질의 종류에 따라 최대로 전시가능한 임계점이 존재하며 2 가지 지질 종류의 PEG 에 대한 페길화 임계점과 입자 표면의 PEG 밀도에 따른 입자 안정성의 차이를 비교하였다. DSPE-PEG 보다 DMG-PEG 의 전시 효율이 약 20 배 정도 높았으며 입자안정성도 페길화 임계점과 함께 증가하는 것을 확인하였다. 페길화의 또 다른 강점으로는 PEG 말단에 표적 물질을 합성하여 나노베지클의 표적 치료제로서 역할을 부여한다는 것을 유효성 평가를 통해 검증하였다. 페길화 나노베지클에 표적 펩타이드를 전시할 경우 세포

흡수가 증가하여 보다 효과적인 약물전달체로 이용될 수 있다. 활용 예로 페길화 나노베지클을 이용하면 장기 점막 투과성을 증진시켜 치료효과를 개선시킬 수 있다. DMG-PEG 가 DSPE-PEG 보다 페길화 효율과 임계점이 높았으며 치료제의 목적에 따라 장점이 달라질 수 있다. 추가 연구로는 PEG 밀도에 따른 체내 순환시간의 변화, 적응증 별 세포 흡수 양상, 적응증 별 in-vitro 와 in-vivo 유효성 평가에 따른 페길화 밀도 최적화가 필요할 것으로 사료된다.

본 연구에서는 인체 친화적인 줄기세포 유래의 나노베지클을 페길화하여 생체안정성을 증대하고 PEG 에 접합된 지질의 종류에 따른 페길화 임계점과 정량적 분석법을 제시한다. 나아가 PEG 말단에 펩타이드를 전시하여 나노베지클의 약리효과가 증대될 수 있음을 유효성을 통해 확인하였다. 또한 PEG 접합 지질의 특성에 따라 페길화의 임계점이 달라질 수 있는 것을 확인하였다. 이 연구 결과는 줄기세포 유래 나노베지클도 치료 부위와 목적에 따라 표면 개질이 가능하고 정량적 분석이 가능하다는 것을 시사한다.

핵심되는 말: 세포외 소포체, 나노베지클, 줄기세포, 표면개질, 체내안정성, 페길화