Functionalization of Extracellular Vesicles

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ABSTRACT

Exosomes, a subpopulation of extracellular vesicles, are involved in intercellular long-distance communication and have great potential as a novel drug delivery system for Central Nervous System diseases like Alzheimer's disease. This disease is characterized by the deposition of the β-amyloid peptide as amyloid plaques and one of the potential treatments for this disease could be based on the use of Neprilysin (NEP), a β-amyloid peptide-degrading enzyme. In order to increase the efficacy of current exosome-based therapies, multiple surface modifications can be made to exosomes for cellular and subcellular targeting. This work aims to optimize methodologies to obtain functionalized exosomes through fusion with synthetic functionalized liposomes. The fusion between unlabelled liposomes and fluorescent labelled liposomes was performed through the freeze-thaw method. The results demonstrated that the functionalization of unlabelled liposomes with extrinsic characteristics was accomplished, and later, the production of hybrid vesicles through fusion with EVs was also achieved. Click chemistry was used for the bioconjugation of molecules such as BDP-azide and PEG-Azide to the surface of liposomes in order to optimize conditions for functionalization of hybrid EVs. The click chemistry reaction between the DBCO groups and the fluorophore BDP-azide was extremely efficient resulting in almost instantaneous ligation of all the DBCO groups to BDP-azide molecules. The reaction with PEG-azide, revealed to be slower. This functionalization was quantified for two different concentrations of DBCO (10μM and 25μM). After 48h, the functionalization efficiencies were respectively, 53.85% and 55.17%, suggesting complete functionalization of the outer leaflet of the liposomes. The potential negative impact of using the freeze-thaw method on NEP enzyme activity was also quantified, demonstrating that the NEP enzyme does not lose significant activity when subjected to the different freeze-thaw cycles while incorporated into the membrane of the EVs. This favourable result suggests that the production of hybrid EV-liposome particles via freeze-thaw induced fusion is a valid and useful method to achieve functionalization of EVs-based particles.

Keywords: EVs (Extracellular Vesicles); Exosomes; Liposomes; Alzheimer; Neprilysin; EV Engineering; Click Chemistry

1. Introduction

In recent years, intense research efforts have been devoted to the study of membrane trafficking by exosomes, due to the potential as biomarkers for diagnosis and as a route for drug delivery.[1] Exosomes might be an alternative to synthetic nanoparticles, solving clearance problems and preventing toxic effects through a more specific and controlled transport of drugs, particularly important, for example, in cancer therapeutics or in treating neurological diseases.[2] Their biocompatibility, no inherent toxicity, stability, low immunogenicity, natural ability to carry intercellular nucleic acids and therapeutic molecules across membranes with low permeability, such as the Blood Brain Barrier (BBB), as well as adjustable targeting efficiency, are some of the characteristics that make exosomes great drug delivery systems.[3,4] The ability of exosomes to overcome the Blood Brain Barrier and offer targeted drug delivery, supports their potential as a novel drug delivery system for Central Nervous System diseases like Alzheimer's. Alzheimer's disease (AD) is a chronical disease associated with the progressive damage of neurons. This disease is characterized by the deposition of the β-amyloid peptide as amyloid plaques and one of the potential treatments for this disease could

be based on the use of Neprilysin (NEP), a β-amyloid peptide-degrading enzyme [5] . In order to increase the specificity of current exosome-based therapies, multiple surface modifications can be made to the exosomes for cellular and subcellular targeting. There are several approaches that can be carried out for this purpose. In addition, active drug loading of exosomes can be achieved by different techniques such as sonication, extrusion, electroporation, chemical-based exosome incorporation with exogenous cargoes or drug conjugation techniques.[6] One of the strategies explored in this work for the functionalization of EVs is their fusion with functionalized synthetic liposomes. Liposomes are composed of one or more lipid bilayers that enclose an aqueous medium. [7,8] The functionalization of EVs by their fusion with functionalized synthetic liposomes will be employed using the physical method of freeze-thaw. This is a straightforward technique used to make hybrid EV-based vesicles from EVs and liposomes. This method disrupts the lipid bilayer by temporary formation of ice crystals. [8] The fusion of functionalized liposomes with EVs combines the advantages of liposome technology and the natural features of EVs, enabling the efficient functionalization of the EVs. The fusion process can be monitored by Förster resonance energy transfer (FRET). FRET, is a physical phenomenon in which a donor fluorophore in its excited state non-radiatively transfers its excitation energy to a neighbouring acceptor.[9] Chemical methods can also be used to directly attach molecules to the surface of exosomes via covalent bonds leading to the functionalization of the exosomes. The conjugation of these molecules to the exosome surface may enable specific interactions of exosomes with target cells. Click chemistry is an ideal technique for the bioconjugation of small and macro-molecules such as nucleic acids, lipids and proteins to the surface of exosomes. Click chemistry is described as the reaction between an alkyne chemical group and an azide chemical group to form a triazole linkage, and is characterized for being a fast and simple technique with high specificity and high yield. [10, 11] In this work, click chemistry will be used as a method for the functionalization of liposomes before fusing them with EVs. The functionalization of liposomes will be performed using the compounds PEG-azide and BDP-azide through click modification for later functionalization of EVs. The impact on exosomes loaded with Neprilysin will be monitored to ensure minimal interference of the protocol with exosome proteolytic activity.

2. Materials and Methods

2.1 Materials

Chemical Reagents - 1-palmitoyil-2-oleoyl-*sn*-glicero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-dibenzocyclooctyl (DBCO PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific, ethanol (EtOH) and chloroform ($CHCL_3$) were purchased from JMGS. Methoxypolyethylene glycol azide (PEG-Azide), Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Milli-Q water was used throughout the work. **Probes -** 1,2-dioleoyl-s*n*-glycero-3-phosphoethanolamine-N-(lissamine Rhodamine B sulfonyl) (DOPE-Rho) and N-(7-Nitrobenzofurazan-4-yl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE-NBD) were both purchased from Avanti Polar Lipids. BODIPY-azide (BDP-azide) was purchased from Lumiprobe. **ECE-1 substrate -** 7-metoxycoumarin-4-yl acetyl (MCA)-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-2,4-dinitrophenyl (DNP)- Lys peptide (ECE-1 substrate) was purchased from Sigma-Aldrich. This peptide has a molecular weight of 1388 g/mol**.** To prepare aliquots of this peptide, the solution was reconstituted in DMSO, originating sample stocks with a concentration of approximately 4 mM. Aliquots were stored at -20ºC. **Neprilysin -** Purified Neprilysin from porcine kidney was purchased from Sigma Aldrich. The enzyme was reconstituted using PBS. Aliquots were stored at -20ºC with a concentration of 500nM. **DNA constructs** - *pCMV3-MME-OFPSpark and pCMV3-MME.* Human CD10/MME ORF mammalian expression plasmid was purchased from Sino Biological. This vector expresses NEP in its host cells and has a size of 6806bp. In the *pCMV3-MME-OFPSpark*, a C terminal OFPSpark (fluorescent protein) is fused to the expressed NEP. The vector is expressed in mammalian cells. Using T7(TAATACGACTCACTATAGGG) as the forward prime and

BGH(TAGAAGGCACAGTCGAGG) as the reverse prime, it was possible to sequence the plasmid.

2.2 Liposome preparation

For the execution of the FRET assays, the liposomes were prepared using POPC and fluorescently labelled lipids (DOPE-NBD and DOPE-Rho). Samples were prepared with 100% POPC, POPC:DOPE-NBD 99:1, and POPC:DOPE-NBD:DOPE-Rho 98:1:1 mol:mol:mol. For the functionalization of EVs with liposomes, the liposomes were prepared using POPC and DBCO. The different concentrations of DBCO used throughout this project are detailed in the results section for each experiment. After mixing in eppendorfs, the solvent from the different lipid stock solutions was evaporated under nitrogen gas flow. The eppendorf's were then left in vacuum overnight for removal of solvent traces. The next day, 1ml of PBS (heated at approx. 70 degrees Celsius) was added to each eppendorf for hydration of lipid films and the solutions were vortexed. Afterwards, the freeze-thaw cycles were performed and all samples were frozen in liquid nitrogen at -196ºC, subsequently fully heated up to 70ºC and then vortexed. This process was repeated for 8 cycles. The next method employed was extrusion. Extrusion is a technique where the liposome suspension is passed through a membrane filter of defined pore size.[12] All three samples were extruded through a polycarbonate membrane filter with 100-nm pores purchased from Whatman. Filter supports with 10mm from Avanti Polar Lipids were also used in this technique. First of all, all membranes were hydrated with PBS, then the extruder was assembled and PBS was passed through the system before any other solution did. POPC, POPC plus DOPE-NBD, POPC plus DOPE-NBD, DOPE-Rho and POPC plus DBCO were extruded 21 times.

2.3 Membrane Fusion

The POPC liposomes without fluorescent labelled lipids were fused with liposomes with DOPE-NBD or with liposomes with DOPE-NBD and DOPE-Rho. The fusion of the membranes of the different liposomes was established using the freeze-thaw method. As mentioned earlier, this method implies that the solutions are frozen in liquid nitrogen at about −196 °C and then are thawed at 70ºC and vortexed after each cycle. For the optimization of the fusion process, different ratios and different number of freeze-thaw cycles were tested. With each ratio, samples would go through 2 ,4 and 8 cycles of freeze-thaw.

2.4 Fluorescence Measurements

The fusion efficiency was evaluated using FRET assays. NBD and rhodamine are an excellent FRET pair and FRET efficiencies can be quantified through measurements of NBD fluorescence intensity. The sample fluorescence was measured using a FP-8500 fluorescence spectrometer (JASCO, Tokyo, Japan). Fluorescence emission spectra of all samples were obtained with excitation of DOPE-NBD at 460 nm. For POPC:DOPE-NBD 99:1 mol:mol samples, a single fluorescence peak is detected at ~ 530nm, corresponding to the NBD fluorescence. For POPC:DOPE-NBD:DOPE-Rho 98:1:1 mol:mol:mol samples, two peaks are observed, at 530 and 580 nm, corresponding to the emissions from DOPE-NBD and DOPE-Rho, respectively. Dilution of DOPE-Rho due to membrane fusion with "empty" liposomes increases the fluorescence intensity at 530 nm and decreases the intensity at 580 nm, as result of lowering FRET efficiencies. The FRET efficiency of the different liposome mixtures was defined as:

$$E = 1 - (I_{DA}/I_D) \qquad (1)$$

where I_{DA} corresponds to the NBD fluorescence intensity of the liposomes marked with both DOPE-NBD and DOPE-Rho, and I_D corresponds to the NBD fluorescence intensity of the liposomes marked with DOPE-NBD. NBD fluorescence intensity was calculated by integrating the signal from 470 to 530 nm, which excludes the signal from rhodamine. The measurements in the fluorescence spectrometer were carried out using 0,5 cm x 0,5 cm width quartz cuvettes at room temperature.

2.5 Absorption measurements

UV-visible absorption spectroscopy measurements were achieved at room temperature using a double beam V- 660 Jasco spectrophotometer (Jasco Corp., Tokyo, Japan). To determine probe and peptide concentrations, the absorption spectra were measured in a 1cm x 1cm or 0,5cm x 0,5cm path length quartz cuvettes (Hellma Analytics) using a bandwidth and sampling interval of 1nm.

2.6 Click Chemistry

The reaction of click chemistry in liposomes was carried out with different ligand concentration and the reaction was monitored at different reaction times, in order to identify optimum reaction conditions. Firstly, the reaction was performed using liposomes labelled with DBCO reacting with BDP-azide, to study the effectiveness of the incorporation of azide-lipids into the membrane of the liposomes. UV-visible absorption spectroscopy measurements were taken right after the addition of the BDP-azide and then at 3h, 6h, 24h and 48h after the beginning of the reaction, depending on the experiment. The samples were kept in constant agitation for the whole reaction process. Later in this project, this same method was used to functionalize liposomes with DBCO, using a different chemical compound PEG-azide. The reaction was performed in the same conditions as described previously. The different concentrations of DBCO, BDP-azide and PEG-azide used throughout this project are detailed in the results section for each experiment.

2.7 Extracellular Vesicles isolation and quantification

EVs were isolated from conditioned medium collected after 48 hours using the Total Exosome Isolation commercial kit (Invitrogen). Cells were seeded at a density of 6 x 10^6 per cm^2 and transfected with pCMV3-MME-OFPSpark plasmid using Transporter 5™ as a transfection reagent. Cells were maintained for 48h in a T75, in DMEM supplemented with 10% Exosome-depleted FBS and 1% PenStrep at the incubator with controlled temperature (37ºC) and humidity and CO2 levels (5%). After 48h, conditioned medium containing EVs was collected and centrifuged for 30 min at 2000xg to remove cells and cellular debris in suspension. Subsequently, supernatant (~14 ml) was transferred to a falcon and mixed with ~7ml of Total Exosome Isolation solution and stored overnight at 4ºC. In the next morning, mixture was centrifuged at 10000xg for 1h (4 ºC). Finally, the pellet containing the EVs was carefully resuspended in PBS to obtain a concentration factor of 40. EV samples were stored at -80ºC.

The approximate concentration of Neprilysin-OFPSpark in the samples was obtained using a spectrofluorometer (HORIBA) using a OFPSpark calibration curve. Fluorescence measurements of OFPSpark were made with excitation at 535 nm.

2.8 Activity assays with ECE1-substrate

The activity of the NEP enzyme when subjected to various freeze-thaw cycles was tested by performing activity assays with ECE-1-substrate. Activity assays with ECE1-substrate were carried out at 37ºC in the microplate reader using the following filters: excitation: 320/10 nm; emission: 370/10 nm. For this assay, four solutions of NEP with PBS buffer were subjected to freeze-thaw cycles (0,2,4 and 8 cycles respectively). The conditions in which this technique was performed were the same as the ones described previously for the freeze-thaw method. After the cycles, NEP was added to 10 µM solutions of ECE1-substrate. Measurements were carried out with 3 replicates for each condition. The samples were added to the microplate right before initiating the measurements. For activity assays using the EVs, measurements were also carried out at 37ºC. EVs loaded with NEP-OFPSpark had a concentration of 4.35 x 10^{10} particles/mL and were diluted to a final concentration of 0.1 nM of OFPSpark. Naive EVs, without NEP-OFPSpark, had a particle concentration of 8.25 x 10^{10} particles/mL.

3. Results and Discussion

3.1. Fusion between unlabelled liposomes and fluorescent labelled liposomes

Before testing the efficiency of the EV-liposome fusion, the fusion between unlabelled liposomes and fluorescent labelled liposomes was performed. Unlabelled liposomes were fused with fluorescent labelled liposomes by the freeze-thaw method. The fluorescently labelled liposomes were prepared by freeze-thawing and extruding an aqueous liposome dispersion comprising POPC and fluorescently-labelled lipids, DOPE-Rho and DOPE-NBD. The set of NBD and Rho-labelled lipid, very commonly used for the analysis of liposome-liposome fusion, were used to evaluate the efficiency of the liposome fusion in terms of lipid mixing ratio, measured using Förster-resonance energy transfer (FRET). The concentration of NBD and Rho-labelled lipids within the liposomes was always 1:100 (molar ratio) of total lipid. The labelled liposomes were mixed with the unlabelled liposomes at volumetric ratios of 1:1, 1:2, 1:5 and 1:10. Liposomes were diluted to a concentration of 5 μM (total lipid), corresponding to a concentration of 0.05 μM of NBD/Rho. To induce membrane fusion, mixtures were frozen in liquid nitrogen at -196 ºC and thawed at 70ºC. Fusion of "empty" liposomes with liposomes labelled with both DOPE-NBD and DOPE-Rho (at the same concentrations as used above for DOPE-NBD only liposomes) was performed. A total of 4 replicates (n=4) were executed and the results are illustrated in Fig.1 as a measure of FRET efficiency. FRET efficiency values are shown relative to the ratio of labelled to unlabelled liposomes and number of freeze-thaw cycles. The FRET efficiency of the different liposome mixtures was defined as $E=1-(I_{DA}/I_D)$, as described before.

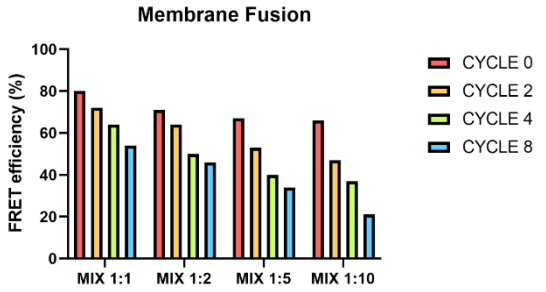

Figure 1 - FRET efficiency of the different liposome mixtures was defined as $E= 1 - (I_{DA}/I_D)$. The decrease in FRET efficiency is a direct indicator of membrane fusion.

Additionally, the FRET efficiency for each ratio of labelled liposomes to unlabelled liposomes, decrease with each freeze-thaw cycle, as made clear from the results in (Fig.2). This happens because there is a greater dilution of the membrane with each cycle made, as further events of liposome fusion occur. The corresponding dilution was estimated for each determined FRET efficiency after freeze-thaw cycles of labeled and unlabeled liposomes (Fig.3). The membrane dilution represents the dilution observed after the fusion of the different liposomes and is an indicator of the efficiency of the fusion method. The results show that as the number of freeze-thaw cycles increases, the membrane dilution increases as well. This was observed for all the different ratios.

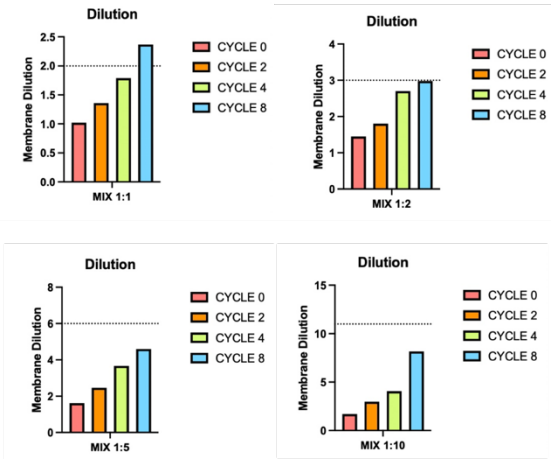

Figure 2 - Membrane dilution compared to the maximum theoretical value (dotted line) achievable upon 100% membrane fusion.

Theoretically, the maximum dilution that could be obtained from the different ratios 1:1,1:2,1:5

and 1:10 was, respectively, 2, 3, 6 and 11, achieved if all of the liposomes had been efficiently fused. When looking individually to each ratio it can be observed that, in the case of the 1:1 ratio the membrane dilution reported for the cycle 8 surpassed the expected theoretical maximum, indicated as a dotted line. This can be explained by measurement errors associated to equipment and inter-experiment variability. In the case of the 1:2 ratio, the fusion cycle that showed the maximum membrane dilution was the cycle 8. Although 4 cycles did not show as much membrane dilution as 8 cycles of fusion, when choosing between the two, 4 cycles of fusion would be preferable, because it produces almost the same membrane dilution and would subject the EVs to less stress. With the labelled: unlabelled vesicle ratios of 1:5 and 1:10, membrane dilution did not reach the theoretical maximum membrane dilution value, but as reported for the other ratios, dilution also increased with the number of cycles performed. The overall results demonstrate that the fluorescent lipids in the labelled liposomes were diluted by the membrane components present in the unlabelled liposomes. A change in the lipid composition of the unlabelled liposomes was achieved with membrane fusion with labelled liposomes. It can be extrapolated that this method can be replicated with EV-liposome fusion with the purpose of engineering EVs with already functionalized liposomes. In summary, these results indicate that the fusion of liposomes with labelled liposomes was effective, and that the functionalization of unlabelled liposomes with extrinsic characteristics was accomplished.

3.2. Fusion between EVs and fluorescent labelled liposomes

After the fusion between unlabelled liposomes and fluorescent labelled liposomes, the fusion between EVs and fluorescent labelled liposomes was tested. EVs were fused with fluorescent labelled liposomes by the freeze-thaw method. The fluorescently labelled liposomes were prepared in the same manner as described previously while EVs were produced and isolated from HEK cells as described in the methods section. Liposomes were diluted to 1μM (BDP/Rho diluted to 0.01 μM). At this concentration, assuming that liposomes of 100 nm are constituted by 1 x 10^5 lipids, an approximated 6 x 10^9 liposomes per mL were estimated using Avogadro's number. EVs were diluted to 10 x 10^9 particles per mL. EVs were mixed with the labelled liposomes at a volumetric ratio of 1:1 and the FRET efficiency was calculated prior to freeze-thaw (cycle 0) and for cycles 2 and 4 (Fig. 5).

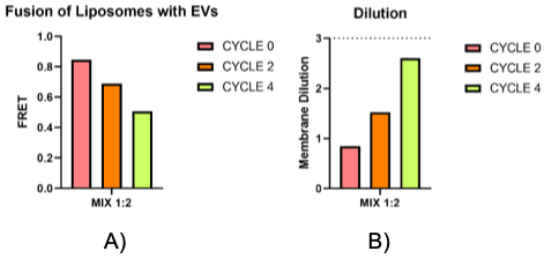

Figure 3 – A) FRET efficiency of the liposome and EVs mixture was defined as E= 1 - (I_{DA}/I_D). The FRET calculation is a direct indicator of membrane fusion. B) Membrane dilution compared to the maximum theoretical value (dotted line) achievable upon 100% membrane fusion.

The results show that the FRET efficiency for the approximated 1:2 particle ratio of labelled liposomes to EVs, decreases with each freeze-thaw cycle, as evidenced from the results in (Fig.5). This happens because there is a greater dilution of the membrane with each cycle, indicating that the fusion of EVs-liposomes was effective. The FRET efficiency for the approximated 1:2 particle ratio of labelled liposomes to EVs was very similar to the FRET efficiency observed for the 1:2 ratio of labelled liposomes to unlabelled liposomes, allowing for the assumption that the optimization of the fusion conditions between labelled liposomes and unlabelled liposomes can be extrapolated for the EV-Liposome fusion, and the ideal conditions would be the same. The corresponding dilution was estimated for each determined FRET efficiency after freeze-thaw cycles of labeled liposomes with EVs. (Fig.5). The results show that, as it was observed for the experiments with labeled and unlabeled liposomes, as the number of freeze-thaw cycles increases, the membrane dilution increases as well. As mentioned previously, the maximum dilution that could be obtained from the ratio 1:2 is 3, assuming that all of the liposomes had been efficiently fused with the EVs. In this particular case, the fusion cycle that showed the maximum membrane dilution was the cycle 4.

The results for the dilution of the 1:2 ratio are once again very similar to the results from the dilution observed with the labeled and unlabeled liposomes, allowing for the assumption that, just like the fusion of liposomes with labelled liposomes, the fusion of labelled liposomes and EVs was effective, and the functionalization of EVs with extrinsic characteristics was accomplished.

3.3. Functionalization of Liposomes

Initially, an azide containing the fluorophore BDP-azide was employed for optimization of the functionalization process. In addition, the functionalization of liposomes was performed with PEG-azide. The liposomes were prepared with POPC and DBCO-PE. By introducing a lipid with a DBCO group in the liposomes composition, the group could be covalently linked to an azide containing molecule. This technique allows for the functionalization of liposomes with different molecules. The group DBCO absorbs light with a maximum at around 290nm, and on the other hand, the product of the DBCO-azide reaction does not absorb light, which allows for the reaction with azide to be followed spectroscopically. Alternatively, the fluorescence of liposomes labelled with BDP-azide can also be used to characterize functionalization efficiency. Different percentages of DBCO lipids were tested, and its modification efficiency was monitored. As it was described previously, the click chemistry reaction in liposomes was executed with different ligand concentration and the reaction was monitored at different reaction times, in order to identify optimum conditions. The first functionalization experiment was performed by adding the fluorophore BDP-azide to the liposomes containing 25 μM DBCO. The experiment was performed with two different ratios (1:1 and 1:4) of DBCO to BDP-azide. UV-visible absorption spectroscopy measurements were taken right after the addition of the BDP-azide and then at 3h, 6h, and 24h after the beginning of the reaction. The samples were kept in constant agitation for the whole reaction process.

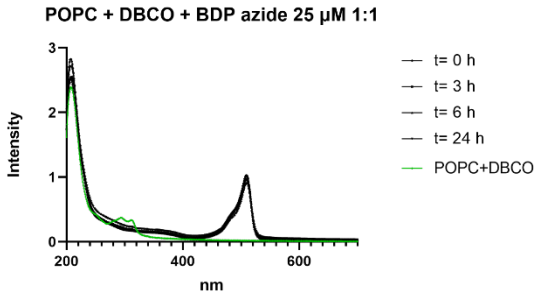

Figure 4 - Absorbance spectra of click chemistry reaction with a 25μM DBCO concentration, a 1:1 proportion of DBCO to BDP-azide and different measurement times.

As it can be observed in Fig.7, the absorbance band of DCBO with a maximum around 290 nm is easily identified from the sample prior to incubation with the azide fluorophore. The absorbance spectra from the samples taken at the different reaction times (t= 0, 3, 6, and 24 hours) are very similar, with near complete elimination of the DBCO band. The band associated to the emission maximum of the BDP-azide at 512 nm can also be observed. The results clearly show that when the BDP-azide is added to the solution, the band associated with DBCO disappear immediately. The same situation was observed in the samples with the 1:4 ratio of DBCO to BDP-azide. The results above suggest that the click chemistry reaction between the DBCO groups and the fluorophore BDP-azide was extremely efficient resulting in all of the DBCO groups ligated to BDP-azide molecules, immediately after the onset of reaction. Such a fast reaction, implies that BDP-azide readily translocate to the inner leaflet of the vesicle and react with DBCO groups located there, which are expected to correspond to roughly 50% of total available. This is not unexpected, since BDP-azide is a fairly hydrophobic molecule.

3.4. Functionalization of Liposomes with PEG-azide

After the functionalization of liposomes with BDP-azide, the functionalization of liposomes was performed with PEG-azide. The liposomes were, once again, prepared with POPC and DBCO. As mentioned previously, DBCO absorbs light with a maximum at around 290 nm. In addition, PEG-azide has no UV-absorption, therefore this molecule does not

interfere with monitorization of the reaction. Airoldi *et al.*[13] suggested that the best results for this reaction would be obtained after 48 h of incubation, and with that in mind, the functionalization of the liposomes with PEG-azide (n=3) in two different concentrations (10μM and 25μM) was performed, and measurements were taken at times 0h, 3h, 6h, 24h and 48h after the beginning of the click chemistry reaction. The samples were kept in constant agitation for the whole reaction process. The results obtained are shown in the Fig.11 and Fig.12.

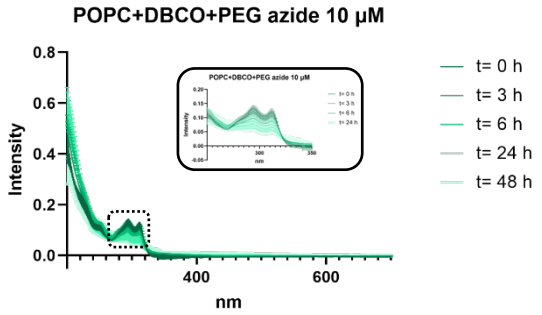

Figure 5 - Absorbance spectra of click chemistry reaction with a 10μM DBCO concentration, PEG azide and different measurement times.

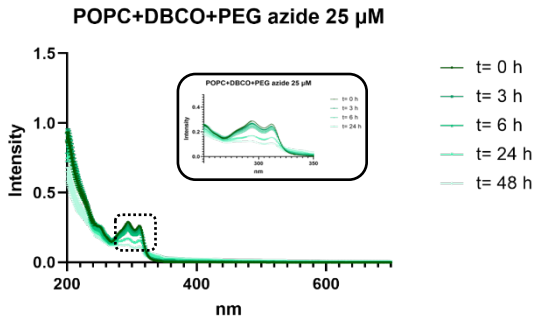

Figure 6 - Absorbance spectra of click chemistry reaction with a 25μM DBCO concentration, PEG azide and different measurement times.

Table 1 - Percentages of functionalization of the liposomes with PEG-azide in two different concentrations (10μM and 25μM) after 3h, 6h, 24h and 48h.

	3h	6h	24h	48h
10μM	7.69 %	7.69 %	38.46 %	53.85 %
25μM	6.90 %	13.79 %	41.38 %	55.17 %

What can be concluded from these reactions is that, unlike the functionalization with the BDP-azide, the functionalization with PEG-azide was dramatically slower. Nevertheless, functionalization can be observed as the band from the DBCO decreases significantly overtime. A more significant decrease in the DBCO absorbance occurs only after 24 hours of reaction, meaning that the functionalization only started to occur many hours after the beginning of the reaction. As shown in table 1, after 48h, around 50% of functionalization is achieved. This is the maximum functionalization expected, since a little less than half of the DBCO groups are facing inwards the liposome membrane and therefore are not available to react with the PEG-azide. The functionalization efficiency with the PEG-azide is very different from the functionalization efficiency with BDP-azide because the fluorophore BDP-azide is an hydrophobic compound that has the ability to translocate to the inside of the vesicle and react with all the DBCO groups.

3.5. The study of the activity of Neprilysin (NEP) with ECE1-substrate

Since freeze-thaw cycles can potentially lead to enzyme denaturation, the study of the activity of the enzyme Neprilysin with ECE-1-substrate after being subjected to freeze-thaw cycles was performed. The ECE-1 substrate is an internally quenched fluorogenic substrate that can be cleaved by NEP and IDE [14,15]. This peptide contains a fluorophore (MCA; FRET donor) that has an emission spectrum that overlaps with the absorbance spectrum of a quencher (DNP; FRET acceptor), also contained in the peptide, resulting in a transference of energy between the fluorophore and the acceptor that quenches the donor fluorescence. When the peptide is cleaved at any position, the separation of the fluorophore and FRET acceptor eliminates FRET, and an increase in the signal is observed. By monitoring the fluorescence intensity of this peptide, it is possible to evaluate the activity of a protease. The maximum fluorescence intensity is reached when there is no more substrate for the enzyme. In this experiment, purified porcine NEP's activity was tested using ECE-1 substrate. This enzyme does not cut peptides with high specificity and always in the same region. This allowed for the study and optimization of the conditions at which the enzyme has more activity using a generic protease substrate such as the ECE-1 peptide. With this method, the ability of NEP to successfully cleave ECE-1 was studied. If, as

mentioned previously, for the fusion of different liposomes, the 1:2 ratio and the cycle 4 were chosen as the optimum conditions for a fusion experiment, it would be crucial to know that this enzyme would still be active in these conditions. In this experiment, four solutions of NEP with PBS buffer were subjected to freeze-thaw cycles (0, 2, 4 and 8 cycles respectively). After the cycles, the ECE1-substrate solution was added to the 4 solutions and measurements were run with 3 replicates for each condition. Four independent experiments (n=4) of activity studies with ECE1-substrate were performed (Fig. 13).

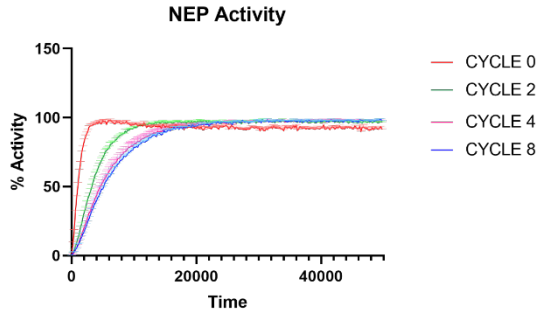

Figure 7 - Study of the activity of the enzyme Neprilysin with ECE-1-substrate after being subjected to the freeze-thaw cycles.

The results from the Fig.13 indicate that, as predicted, MCA fluorescence intensity increases with time, confirming that NEP has indeed activity against ECE-1 substrate. The results also show that as the number of cycles increases, the enzyme neprilysin loses some of its activity, therefore diminishing the final concentration of cleaved MCA in solution, which results in a lower value of MCA fluorescence intensity. This result is to be expected as the freeze-thaw method implies great variations in temperature that could affect the enzyme's activity due to denaturation. Nonetheless, the NEP enzyme retained significant activity after 8 cycles of freeze-thaw, which is very positive, as a relatively large number of freeze-thaw cycles can be used during fusion and functionalization experiment, without loss of most NEP activity. Following the study of the activity of the enzyme Neprilysin with ECE-1-substrate after being subjected to the freeze-thaw cycles, the same experiment was performed but with EVs engineered to present Neprilysin. The purpose of this second experiment was also to understand the potential negative impact of freeze-thaw on enzyme activity and to compare this impact when the enzyme is free or incorporated in the EVs.

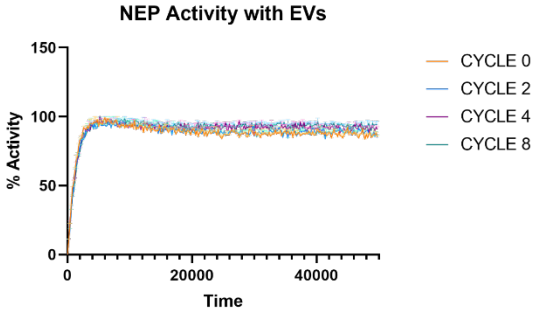

Figure 8 - Study of the activity of the enzyme Neprilysin in EVs with ECE-1-substrate after being subjected to the freeze-thaw cycles.

The results from the NEP activity studies made with EVs (n=2) are shown in Fig.14 and they present a much more consistent activity throughout the cycles than the purified free NEP enzyme, suggesting no impact whatsoever of the freeze-thaw cycles on enzyme activity. This could be due to NEP being more protected from external conditions by being incorporated in the membrane of EVs, which reduces the probability of denaturation during freezing.

4. Conclusion

Regarding the fusion between unlabelled liposomes and fluorescent labelled liposomes, it can be concluded that the freeze-thaw technique was effective and that the functionalization of unlabelled liposomes with extrinsic characteristics was accomplished. This was the first step into the functionalization of liposomes and exosomes. The fusion between EVs and fluorescent labelled liposomes was also tested and the results showed that this fusion was effective, confirming the possibility of using this methodology to engineer hybrid EVs with already functionalized liposomes. The functionalization of liposomes by click chemistry reaction between the DBCO groups and the fluorophore BDP-azide was extremely efficient resulting in all of the DBCO groups ligated to BDP-azide molecules. After witnessing the live click chemistry reaction, it could be concluded that part of the reason why the reaction was so effective was because the BDP-azide is hydrophobic, which might lead to the ability to

translocate the phospholipid bilayer membrane of the GUV's, associate to the membrane and ligate to the DBCO groups that are directed inwards of the vesicle, that otherwise would be unreachable. The functionalization of liposomes with PEG-azide showed considerably lower efficiency than functionalization with BDP-azide, and a much longer reaction time was necessary to achieve significant functionalization. The introduction and functionalization of the DBCO group indicates that any peptide conjugated with an azide group can be bound to liposomes and then fused to EVs, opening a world of possibilities regarding EVs functionalization. The last step of this thesis consisted in activity studies of the enzyme NEP, which presented significant activity when subjected to multiple freeze-thaw cycles. NEP activity from the enzyme within EVs was shown to be much more resistant to freeze-thaw cycles than the activity of soluble purified enzyme. The fact that NEP did not lose activity when subjected to the different freeze-thaw cycles while incorporated in the EVs membrane, suggest that if EVs bearing NEP in their membrane were functionalized by fusion with liposomes, the enzyme would still be active after the cycles and could potentially exert its capacity as a β-amyloid peptide-degrading enzyme preventing pathogenic changes in the brain. In this way, production of hybrid EV/liposome particles through the freeze-thaw technique is ideal to achieve further functionalization of NEP-loaded EVs, which could be employed in therapeutic applications in the context of Alzheimer's disease. The same methodology is likely to be successful in achieving EV functionalization for other applications. The simplicity of the procedure is a considerable advantage over alternative methods to achieve functionalization. Through control of liposome composition, extraordinary control of final EV functionalization pattern is possible. However, it is not entirely clear yet if the hybrid vesicles produced as described here retain all of the advantageous properties of EVs and further research will be necessary to clarify this issue.

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