Extracellular vesicles from a natural source for tailor-made nanomaterials

VES4US

[D4.1] Functionalization strategy report

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1. INTRODUCTION

The functionalization of extracellular vesicles, which is the main aim of WP4, has been an area of research already for the past years. We now aim to identify the most promising approaches to be applied for the engineering of natural source-derived EVs in the VES4US project. Generally, there are two main strategies for the introduction of functional structures on the EV surfaces: 1) Engineering of the parent cells or 2) direct chemical/physical modification of the isolated EVs.[1, 2] Parent cell engineering involves either the genetic manipulation to introduce e.g. specific membrane proteins or metabolic labelling, meaning that non-native metabolites are fed to the cells who then integrate them into all cellular membranes. In our project, parent cell engineering might be more feasible at a later stage as soon as the preferred species of the identified natural source for EV production are selected and the conditions for optimal yield are optimized. Currently, we therefore focus on the direct chemical modification of vesicle surfaces using established chemistry for bio-orthogonal reactions.
For covalent functionalization, it is essential to choose suitable reaction conditions because factors like elevated temperatures or large amounts of organic solvents can lead to vesicle aggregation and damage to membranes and membrane proteins. A possible mild reaction is therefore the so-called “click-chemistry”. Click reactions are highly selective and can be performed in aqueous environment without the need for excessive reaction conditions. The alkyne-azide click reaction has already been successfully used for EV functionalization in the presence of a copper catalyst (CuAAC).[3, 4] However, copper is known for its deleterious effects on cells, so that it has to be removed from the system after the reactions. Often, this cannot be ensured reliably, so that it is preferred to avoid its presence completely. Alternatively, the strain-promoted alkyne-azide click reaction (SPAAC) might be employed for EV functionalization.[5-8] In this case, no copper catalyst needs to be present as sufficient reactivity is guaranteed by the introduction of a strained alkyne.

In the present deliverable, we propose a functionalization strategy for extracellular vesicles, which can directly be applied to the EVs obtained from the selected natural source without the need for parent cell engineering (metabolic labelling). We suggest to employ NHS chemistry for the introduction of strained alkyne groups followed by a copper free click reaction with azidated proteins.

2. PROPOSED FUNCTIONALIZATION STRATEGY

2.1 ESTABLISHMENT OF LIPOSOME MODEL SYSTEM FOR FUNCTIONALIZATION PROOF-OF-CONCEPT

In the VES4US project, the functionalization of extracellular vesicles is a key point for their subsequent application, so that the functionalization strategies must be optimized carefully. Thus, we have decided to perform all functionalization reaction steps including purification and characterization efforts on a model system, before working with the selected natural source-derived EVs. As the EVs are obtained in smaller amounts in the beginning of the project, material can be saved for a thorough characterization of the EVs before starting the functionalization work. Accordingly, in a first step the setup of a suitable model system is proposed. We have decided to mimic the properties of EVs by producing liposomes, which also consist of lipid bilayers. The membrane properties can be tuned by varying the membrane composition. Thus, we can optimize the stability of the liposomal system. The initial starting composition for the liposomes as shown in Figure 1 was selected to be a 1:1:1 molar ratio of 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), L-α-phosphatidylcholine (egg PC) and cholesterol. Most important in this case is the selection of a phospholipid introducing reactive functional

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groups, which are similarly present also on EV surfaces. As it is known that EVs feature membrane proteins, we rely on the presence of primary amino groups (-NH_2) mostly from lysine residues, as they are most reactive compared to other biological functional groups. For this reason, DOPE was chosen as a component of the liposomal system, allowing the synthesis of NH_2-terminated liposome surfaces. The liposomes are produced via thin-film hydration and subsequent extrusion (synthetic procedure see Figure 1), which is a common method to yield both liposomes and polymersomes with a narrow size distribution. This way, the liposomes can be synthesized in the desired physiological aqueous buffer conditions (e.g. in PBS buffer, pH 7.4) or any other aqueous medium, which is used for EV isolation/purification. The liposome size can easily be tuned to match the size of obtained EVs by adjusting the pore size of the membranes used for extrusion.

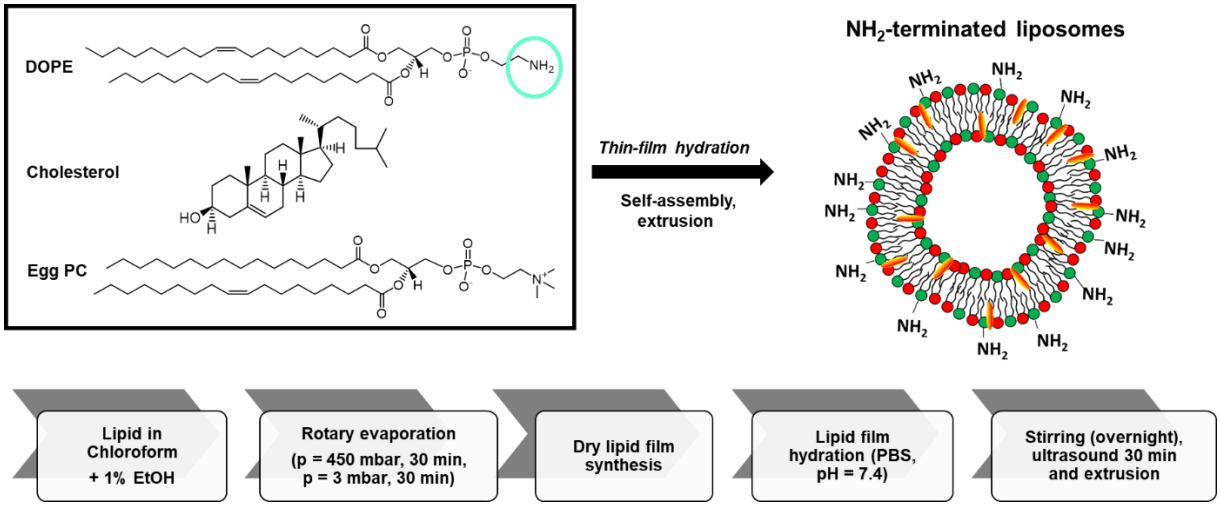

Figure 1. Proposed synthesis scheme to produce liposomes as extracellular vesicle model systems.

To enable precisely controlled surface functionalization after the liposome synthesis, the number of accessible reactive amino groups needs to be determined. Then, the compounds used for further coupling reactions can be adjusted to specific molar ratios. For the quantification of primary amino groups, we propose to use a fluorescamine assay. The assay is based on the reaction of fluorescamine with primary amines to yield a fluorescent product, which can be detected in plate reader measurements (see Figure 2). After obtaining a calibration curve from different concentrations of hexylamine, the exact number of amino groups in a given volume of liposome sample can be calculated. In the same way, this assay can be employed for the detection of amino groups in EV samples with the only difference being the position of the amino groups, which will also be part of the characterization methods proposed for WP3.

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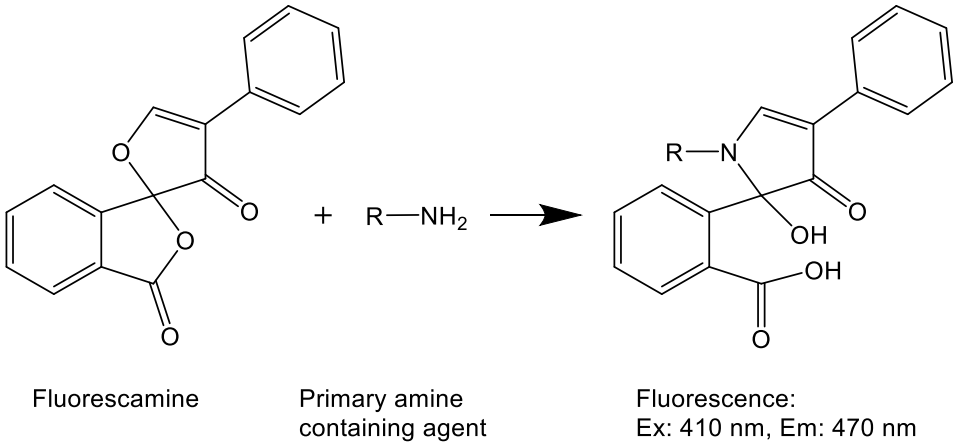

Figure 2. Reaction scheme of the fluorescamine assay applied for NH_2 group quantification.

2.2 INTRODUCTION OF BIO-ORTHOGONAL REACTIVE GROUPS ON VESICLE SURFACES

Once the number of accessible reactive primary amino groups in a given liposome or EV sample is defined, they will be reacted with dibenzylcyclooctine (DBCO)-PEG_4-NHS ester to form a stable amide bond (see Figure 3). This way, amine groups will be converted to strained alkyne groups, which selectively only further react with azide moieties in a [3+2] cycloaddition.[9] As azide groups do not occur in biological molecules naturally, the strategy allows for a bio-orthogonal functionalization reaction. The introduced PEG (polyethylene glycol) spacer enables better accessibility of the alkyne on the vesicle surface. The reaction can be performed with only minimal amounts of organic solvent needed for the solubilization of the DBCO-PEG_4-NHS ester, which is then added to the vesicles in physiological aqueous conditions. Additionally, the reaction does not require heating of the sample, so that potentially present proteins will not be denatured.

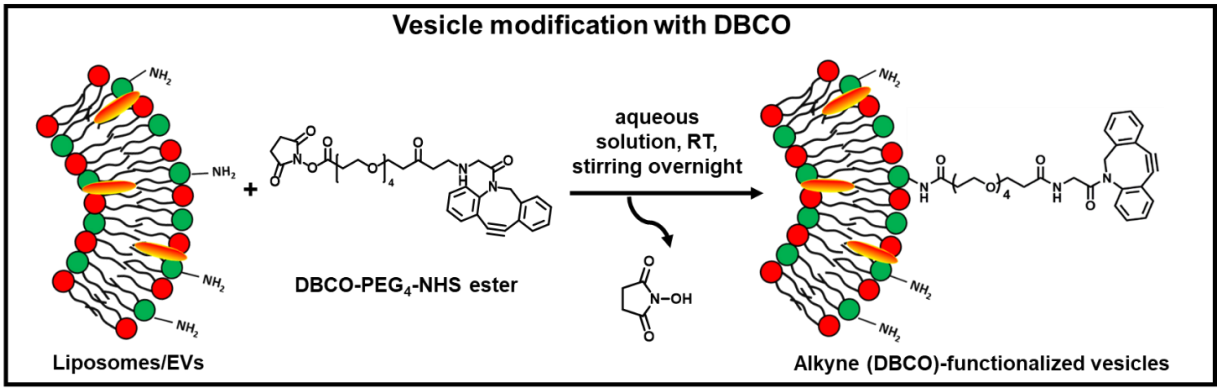

Figure 3. Proposed reaction scheme for the introduction of alkyne groups on NH_2-terminated vesicle surfaces.

After coupling of the DBCO groups onto the vesicle surfaces, the vesicles will be purified by normal centrifugation (liposomes) or centrifugation through spin filter units (EVs) to remove the reaction product succinimide and the introduced organic solvent. Applying spin filters further enables concentrating the EVs as needed. Then, the presence of DBCO groups will be qualitatively verified by Fourier-Transform infrared spectroscopy (FTIR). Subsequently, quantification of the DBCO groups will be carried out in a similar manner as proposed for surface amino groups: In an anthracene-azide assay, the reactivity towards azide functionalized molecules will be exploited to couple anthracene-azide via the strain-promoted alkyne-azide click reaction (SPAAC) (see Figure 4).

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This reaction is very fast and results in a highly fluorescent product, which again can be quantified in plate reader measurements. Like this, also the number of DBCO groups can precisely be determined.

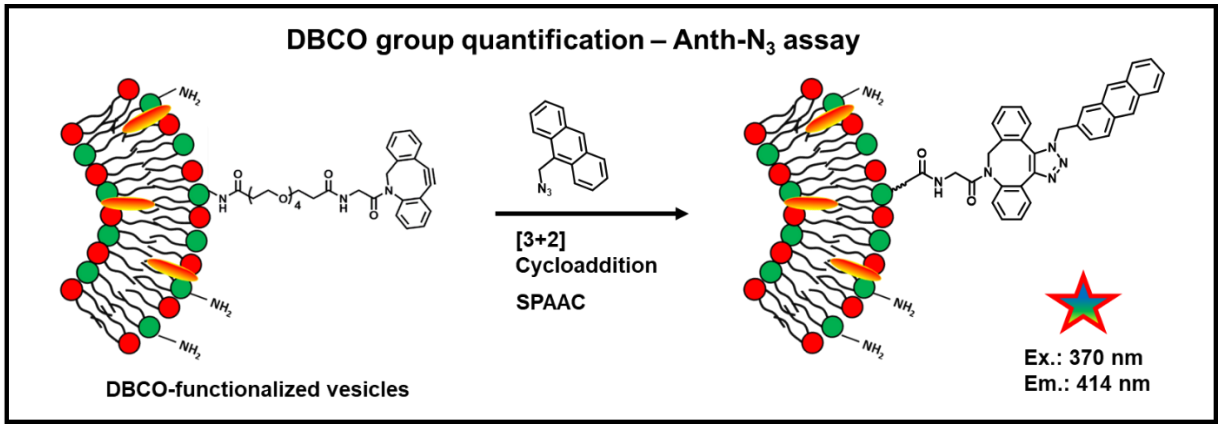

Figure 4. Reaction scheme of the anthracene-azide assay applied for alkyne (DBCO) group quantification.

2.3 MODIFICATION OF FUNCTIONAL BIOMOLECULES/LIGANDS

For the VES4US project, we currently aim at coupling functional proteins onto the surface of EVs. These protein functions can range from the decrease of unspecific cellular uptake ("stealth effect") to active targeting of cell surface receptors. Our functionalization strategy currently focuses on the attachment of antibodies as well as apolipoproteins. Antibodies can be selected for a wide range of potential receptor targets, but of special interest for us are antibodies targeting dendritic cells. The targeting of dendritic cells and other cells involved in the human immune response is a major aim in tumour immune therapy. Thus, for the first coupling approach we have selected the dendritic cell antibody CD11c. For the functionalization of antibodies, NHS-chemistry is most widely used in literature.[10-12] In that case the functional group is attached on the lysine residues or the N-terminus of the antibodies. However, this strategy is highly unspecific as lysine residues are distributed over the whole antibody including the antigen binding sites, where also the N-termini are located. Functionalization along the antigen binding sites thus decreases antibody functionality and recognition. Therefore, we propose a site-selective approach, which is based on the modification of N-glycans located on the asparagine (Asn-297) residues in the antibody Fc fragment. In a two-step protocol, first galactose is removed enzymatically from the sugar-trees, followed by attachment of an azide-labeled sugar derivate (GalNAz, N-azidoacetylgalactosamine-tetraacylated) via a galactose-specific transferase (see Figure 5). This method was firstly developed for site-selective radiolabelling of antibodies and subsequently applied for engineering antibody-drug-conjugates.[13-15]

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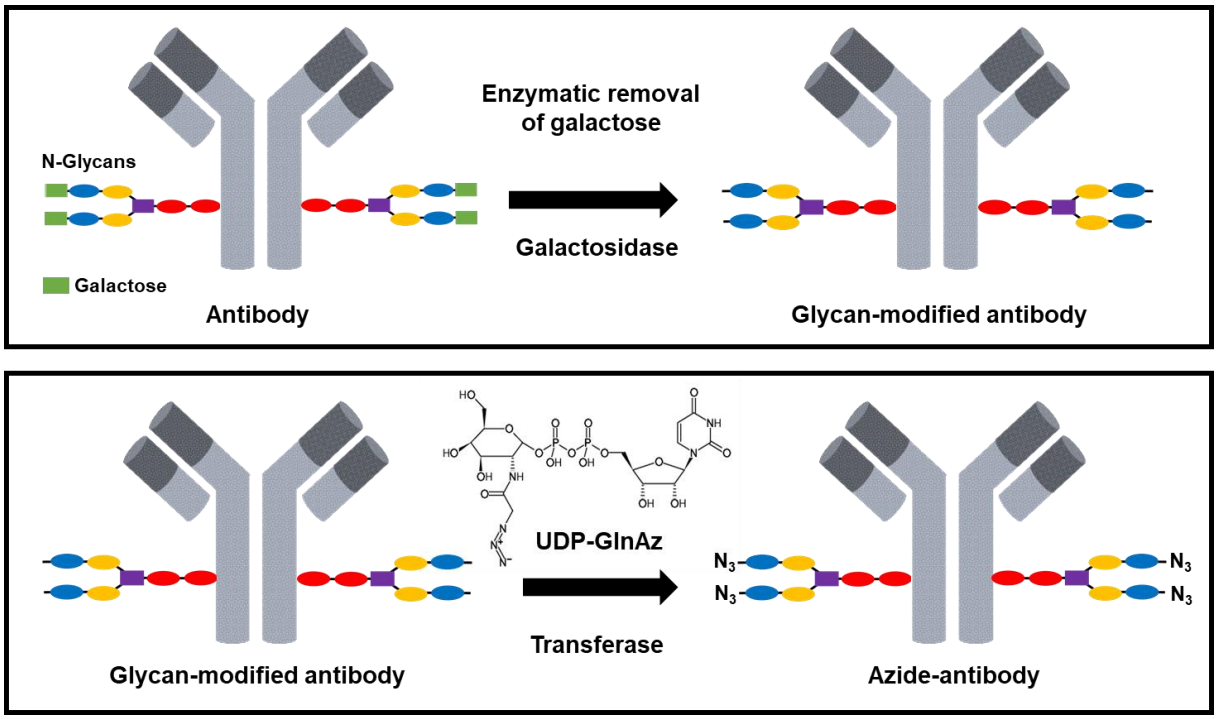

Figure 5. Site-specific azidation of antibodies.

As a second approach, the azidation of apolipoproteins will be performed. In this case, NHS-chemistry was selected as it is not clear whether certain binding motifs for further recognition. Recently, it was reported that apolipoprotein E and A1 could potentially mediate nanocarrier/protein transport over the blood-brain-barrier (BBB).[16, 17] In other studies, nanoparticles coated with polysorbate 80 were also reported to cross the BBB.[18-20] This could potentially have been a result of adsorbed apoliporoteins as polysorbate contains PEG units, which were shown to attract apolipoproteins in general.[21, 22] Additionall, it was found that apolipoprotein J (clusterin) could enhance the transport of amyloid beta-protein over the BBB.[23] The mechanism behind these observations is still not clear, but is suggested that receptor-mediated endocytosis/transcytosis might be responsible for the effect. We would like to use different apolipoproteins to study their targeting properties further. Additionally, we have observed in our studies that especially apolipoproteins induce a stealth effect but dramatically decreasing unspecific cellular uptake.[21] Therefore, we utilize the same chemical reaction as also employed for the surface modification of amino groups on liposomes and EVs (see Figure 6). In this case, though, the NHS ester bears an azide group instead of a strained alkyne. This reaction can again be performed in aqueous solution and does not require further heating. The amount of introduced functional groups again needs to be adjusted depending on the number of available amino groups. So also here, we perform a fluorescamine assay for quantification of amino groups per apolipoprotein. Successful azidation will then be proven by subsequently reacting the azide groups with DBCO-terminated PEG chains ($M_w \approx 5000$ g mol^{-1}) and determine the changed protein molecular weight via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Ultimately, for successful covalent attachment of the apoliporoteins on the vesicle surfaces only one reactive azide group per protein is required. Therefore, the apolipoprotein modification will be optimized to introduced the lowest possible alteration into the protein structure with the smallest possible number of attached groups that still yields successful click coupling.

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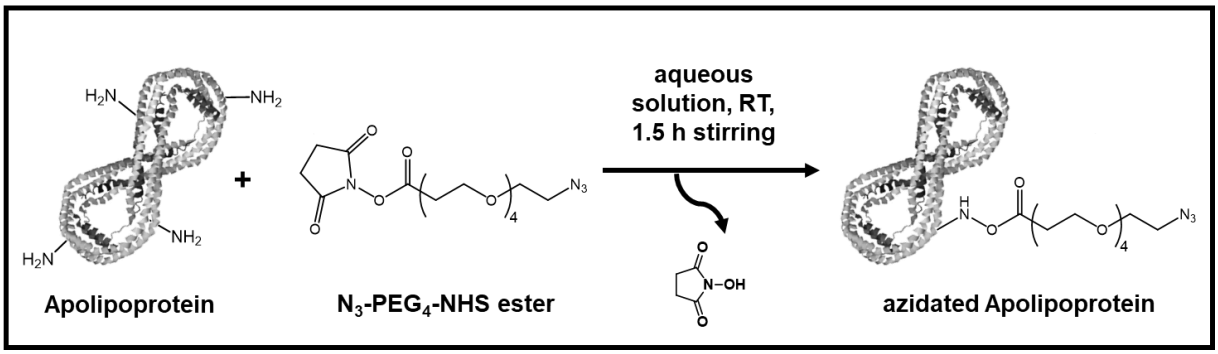

Figure 6. Azidation of apolipoproteins.

2.4 CLICK CHEMISTRY APPROACH FOR LIGAND COUPLING ON VESICLE SURFACES

After obtaining both functionalized vesicle surfaces (DBCO groups) and functionalized proteins (azide groups), the coupling reaction will be performed based on the click chemistry approach as described for the anthracene-azide assay. The alkyne groups specifically react with azide moieties in a [3+2] cycloaddition. In this reaction, no catalyst is required because of the strained alkyne system. The strained ring increases the alkyne reactivity, which normally can only be achieved by utilizing Cu (I) as a catalyst (strain-promoted alkyne-azide click reaction - SPAAC). As such, known deleterious effects of copper in biological systems can be avoided. The reaction is highly efficient (quantitative) and bio-orthogonal.

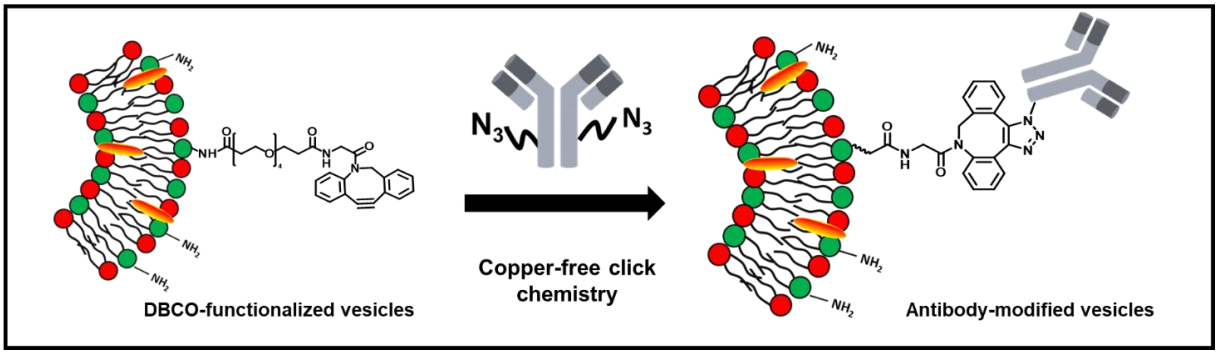

Figure 7. Reaction scheme for the strain-promoted alkyne-azide click reaction (SPAAC) to attach antibodies on the vesicle surface.

After the coupling reaction, purification from unreacted excess protein needs to be performed. Here, we propose to utilize the same purification procedures as introduced for purification after DBCO functionalization. In the case of liposomes, the liposome-protein complexes can be centrifuged and washed. For the EV samples, spin-filters will be applied, although with a higher molecular weight cut-off to enable passing of the proteins through the filter. After purification, the obtained conjugates will be characterized by a flow-cytometry based assay, where fluorescently labelled secondary antibodies bind to the surface immobilized proteins (Figure 8). Then the conjugate can be detected in the flow cytometer, while smaller unbound proteins are not large enough to yield false positive results.

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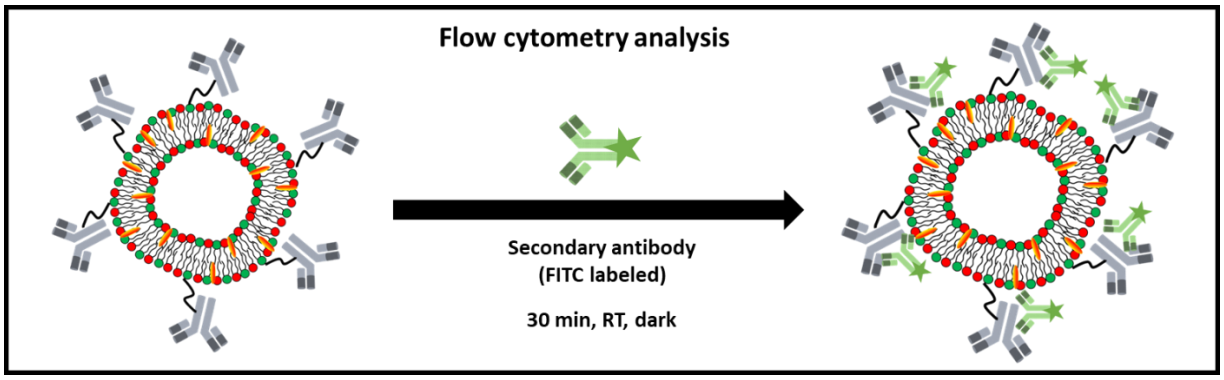

Figure 8. Principle for detection of coupled proteins on vesicle surfaces: Flow-cytometry based fluorescence measurements.

3. ALTERNATIVE STRATEGIES FOR FURTHER CONSIDERATION

The functionalization strategy presented above will be the preferred procedure for straightforward vesicle surface modification. However, in case of insufficient availability of e.g. amino groups, or other difficulties concerning direct surface chemistry, we are proposing an alternative strategy for vesicle functionalization. Based on the fluidity of both liposome and EV membranes, a fusion of both systems is feasible.[24, 25] In that case, additional functional groups could be introduced as long as they are present on the liposome surface. The fusion of liposomes and EVs might be performed by co-extrusion, where vesicle membranes are disrupted by shear force and new vesicles form subsequently by re-organization of the membrane fragments. At the same time, this approach could also be explored for the loading of cargo inside the EVs. Initially, however, we will focus on direct chemical surface functionalization.

4. CONCLUSION

It is anticipated that the presented functionalization strategy allows straightforward surface modification with biomolecules such as proteins for circulation improvement and targeting ability of the EVs as nanocarrier systems. The use of a liposomal model systems allows us to establish the modification concept and optimize the individual steps in terms of reaction conditions, purification and characterization, before working with the EV systems. After the proof-of-concept, the steps can then be transferred to the obtained EV samples. This will be done in close feedback with all partners involved in the EV characterization (WP 3) to ensure that the functionalization is performed on thoroughly analysed samples.

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