CHAPTER 6

Surface Engineering of Biomembranes with GPI-Anchored Proteins and its Applications

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Abstract: This chapter covers the use of glycosylphosphatidylinositol (GPI)-anchored proteins for surface modification of diverse types of biomembrane covered entities ranging from viruses and virus-related particles (section 1), to cells (section 2) and other natural and engineered micro- and nano-scaled particles (section 3). The aim is to present and review state-of-the-art research in this area and to discuss the future direction of GPI painting technology relating to applications in research, biotechnology and biomedicine.

GPI-ANCHORED PROTEIN MODIFICATION OF VIRUSES, VIRAL VECTORS AND VIRUS-LIKE PARTICLES

Background

Viruses do not only cause a large range of pathological conditions but are also used as templates for the generation of biotechnological devices such as viral vectors (VVs) and virus-like particles (VLPs) and commonly used systems derive from adeno-, retro- and lentiviral species. VVs are genetically modified virus particles designed to deliver specific genetic information to cells. This is especially useful in a research setting, when foreign proteins need to be expressed long-term in eukaryotic cells or in gene therapy, where foreign DNA is substituting incomplete or inactive endogenous copies. VLPs contain the structural elements of viruses; however the viral genome and/or functional properties are partly or completely absent. Thus, VLPs cannot infect cells like the viruses or VVs. VLPs are used as particulate carrier systems to provide *e.g.* strong immunogens in vaccine development [1] or a more localised distribution of otherwise soluble agents [2].

Viruses are grouped into two categories based on their structural composition. Enveloped viruses (EV) consist of the genetic material encased in a protein derived core which is in turn surrounded by a lipid bilayer membrane – the envelope. „Naked“ viruses lack such envelope-structures (see Table **1** for a list of enveloped virus families). Presence or absence of envelope structures strongly influence the infectious cycle of viruses and as a consequence therapy strategies differ. One interesting element of virus biology is the apparent association of both enveloped [3-18] (Table **1**) and non-enveloped viruses [19-22] with specific membrane microdomains or lipid rafts.

Although the concept of membrane microdomains and membrane heterogeneity is widely accepted now, the details remain elusive [23-26]. They are proposed as sites for entry and exit of a number of different virus species (for reviews see [27-29]). Furthermore, proteins post-translationally modified with a glycosylphosphatidylinositol-(GPI)-anchor are also not only predominantly localised at the cell membrane but enriched in membrane microdomains. Consequentially, enveloped viral particles can incorporate GPI-anchored proteins when exiting from cells. This has been specifically studied in retroviruses. For example, human immunodeficiency virus 1 (HIV-1) particles carry cell membrane-derived molecules of GPI-anchored CD59 and CD55 after exit from cells [30-32]. This mechanism is beneficial for the virus because CD55 and CD59 inhibit complement activity and can thus protect viral particles from this branch of innate immunity [30-32]. Another important aspect of the biology of GPI-anchored proteins in this context is the capability of purified extracts to re-insert into lipid membranes. This process is mediated by the lipid residues of the GPI-anchor. The re-insertion occurs not only into eukaryotic cells [33,34], but also into the envelopes of viral particles [35] and was described for native GPI-anchored proteins, as well as for proteins that acquired a GPI anchor following genetic modification [36,37]. This phenomenon, termed “painting”, together with the co-localisation of enveloped virus exit and GPI-proteins at membrane microdomains provides the framework for the surface modification of enveloped viruses as described here (Fig. **1**). Both aspects require the presence of lipid structures surrounding the virus particles, thus only enveloped viruses can be modified.

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Table 1: Enveloped viruses and membrane microdomain-associated exit. The table shows the families of enveloped viruses with relevant examples, which may be modified by GPI-anchored proteins. Crosses indicate association of the viral budding processes with membrane microdomain structures. References pertaining to these are given.

FAMILY	EXAMPLE	LR/EXIT	REFERENCE
Herpesviridae	Epstein-Barr virus (EBV)		
	Herpes simplex virus type 1 (HSV-1)		
Hepadnaviridae	Hepatitis B virus		
Poxviridae	Vaccinia virus		
Togaviridae	Rubella virus		
Arenaviridae	lymphocytic choriomeningitisvirus		
Flaviviridae	West-Nile virus		
	Dengue virus		
Orthomyxoviridae	Influenza virus	X	3-5
Paramyxoviridae	Measles virus	X	6.7
	Respiratory syncytial virus (RSV)	X	8-10
	Sendai virus	X	11
Bunyaviridae	Hanta virus		
Rhabdoviridae	Vesicular stomatitis virus (VSV)	X	12
	Rabies virus		
Filoviridae	Marburg virus	X	13,14
	Ebola virus	X	13-15
Coronaviridae	severe acute respiratory syndrome (SARS) associated Corona virus		
Bornaviridae	Borna disease virus		
Retroviridae	Human immunodeficiency virus (HIV-1)	X	16,17
	Murine leukemia virus (MLV)	X	18

State of the Art

Modification of Viral Particles during Cell Exit

GPI-anchored proteins are enriched in membrane microdomains, which also may function as exit sites for different virus species (see Table **1**). This association was successfully used to achieve incorporation of the GPI-anchored protein into the lipid envelope of VVs or VLPs [2,38,39], predominantly derived from retroviral particles. Co-transfection of plasmid vectors carrying genes for the production of retroviral vectors with constructs expressing the GPI-anchored proteins or super-transfection of pre-existing virus producing cell lines leads to the formation of viral particles decorated with GPI-anchored molecules on their envelopes (Fig. **1**, left). These particles acquire novel properties as a consequence of the incorporation of the GPI-anchored protein, *e.g.* super-transfection of the murine retroviral producer cell line PALSG/S with the human GPI-anchored protein CD59, yields viral particles that are resistant to the activity of complement in human serum [38]. These results suggested for the first time that incorporation of recombinantly expressed GPI-anchored proteins into the envelopes of VVs is possible and that these modifications can be useful for gene therapy approaches.

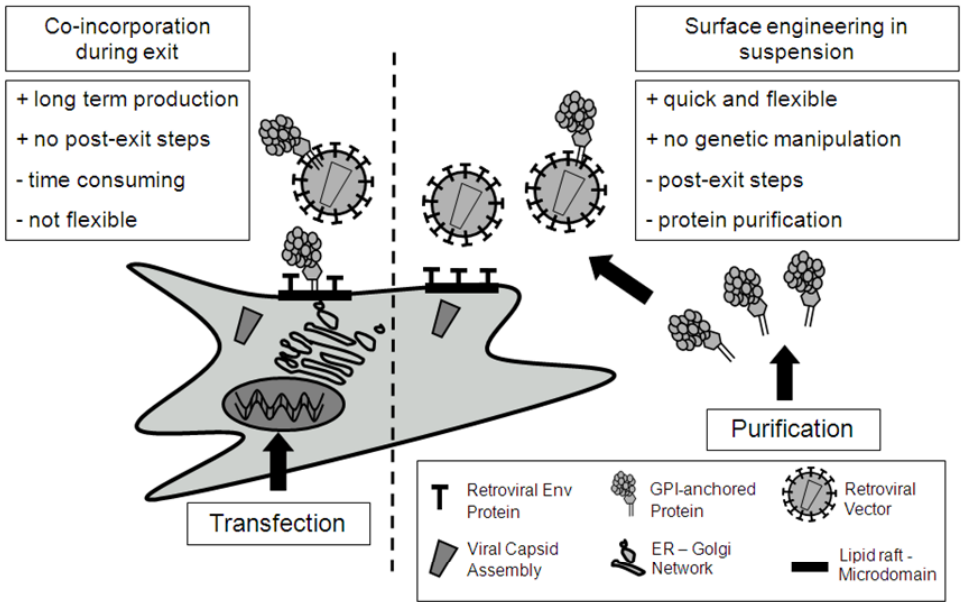

Figure 1: Modification of enveloped viruses, viral vectors and virus-like particles with glycosylphos-phatidylinositol-anchored proteins. Two different pathways are employed to engineer the surface of enveloped viral vectors. Transfection of virus producing cells with

constructs encoding for GPI-anchored leads to co-incorporation during exit from the cells. Alternatively, purified GPI-anchored proteins incubated with enveloped viruses, VVs and VLPs will insert into the virus envelope.

In two more recent studies, co-transfection approaches successfully generated VLPs displaying GPI-anchored molecules from mammalian2 or insect cells [39]. In both cases, recombinant GPI-anchored proteins were generated from different cytokine species i.e. interleukin-2 (IL-2) and granuolocyte-macrophage colony stimulating factor (GM-CSF) and tested for their functional properties. Kueng and co-workers demonstrated that the GPI-anchored cytokines can elicit cellular responses such as differentiation and proliferation with similar efficiency as their soluble counterparts; and Skountzou and co-workers showed that GPI-anchored cytokines engineered onto VLPs based on simian immunodeficiency virus (SIV) can be used to enhance immunogenicity of the VLPs in immunisation studies. Both approaches resulted in a modulation of immune responses provoked by the displayed GPI-anchored cytokine [2,39]. The major advantage of the transfection/co-localisation approach is that stable transfection of (retroviral) producer cell lines co- or super-transfected with endogenous or recombinant GPI-anchored proteins can provide a long-term, reliable source of modified viral particles with high reproducibility. Additionally, no post-exit steps that may reduce infectivity of VVs are required.

Modification of Viral Particles in Suspension

Transfection of producer cell lines is not the only possibility to use GPI-anchored proteins for modification of VVs. A process known as cellular "painting" [33,36] describes that GPI-anchored proteins, when extracted and purified from cells are re-inserted after incubation with eukaryotic cells and localisation is once again conferred to the plasma membrane.

This direct protein engineering or "painting" of enveloped viral particles occurs similar to cell painting when concentrated virus suspensions are incubated with purified GPI-anchored proteins (Fig. **1**, right). This was first described for the GPI-linked model protein CD59his which associates to viral vectors based on MLV and HIV-135, as well as feline herpesvirus (data unpub-lished). The association is specific and painted virus particles remain infectious after insertion of the GPI-linked protein, albeit at reduced efficiencies caused predominantly by the duration of the painting process, rather than the actual incorporation of GPI-anchored molecules into the viral envelope [35]. Estimates of the number of GPI-anchored proteins painted onto retroviral particles were in the range of the numbers observed for Env molecules per virion [35] and is thus similar to that achieved after incorporation of hybrid proteins produced in co-transfection experiments [39]. The main advantage of this approach is its flexibility. Different GPI-anchored proteins can be attached to a range of enveloped viral particles without repeated genetic manipulations of the virus-producing cells. This also means a considerable gain of time, compared to transfection-based methods. Additionally, the amounts of protein deposited at the viral surface may be controllable and only a limited amount of information about the genetic requirements of the virus is necessary for modification.

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PROTOCOL 5

GPI Painting of Viruses, VVs and VLPs:

1. *Prepare concentrated virus stock from desired producer cell lines and purify desired GPI-anchored protein e.g. by affinity chromatography.*
2. *Incubate concentrated virus stocks with GPI-anchored proteins at desired concentration (5-50 µg/ml test range suggested for first trial) for more than 3 hr at 37°C under constant shaking.*
3. *Remove not associated GPI-anchored proteins from virus sample e.g. by ultracentrifugation or ultra-filtration protocols.*
4. *Analyse virus for presence of virus particles and GPI-anchored protein prior to application.*

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Potential Applications

GPI-anchored proteins are useful for the modification of enveloped viral vectors using either of two profoundly different strategies: (i) transfection of viral producer cell lines and (ii) direct protein engineering of viral particles by painting (Fig. **1**). Viral painting may be the method of choice for modification of enveloped viral particles in all situations where a degree of flexibility is favourable, *e.g.* in response to genetic heterogeneity in gene therapy

approaches or in response to high antigen variability for vaccination, and genetic modification of virus producing cell lines is difficult *e.g.* when applying toxic proteins or when handling genetically or biochemically poorly defined virus species are the targets of modification. Three main fields of interest for application are labelling of viral particles *e.g.* for capture or diagnosis, modulation of virus-host interactions *e.g.* immune-modulation for vaccine development and modification of virus binding properties *e.g.* for targeted delivery of VVs (Fig. **2**).

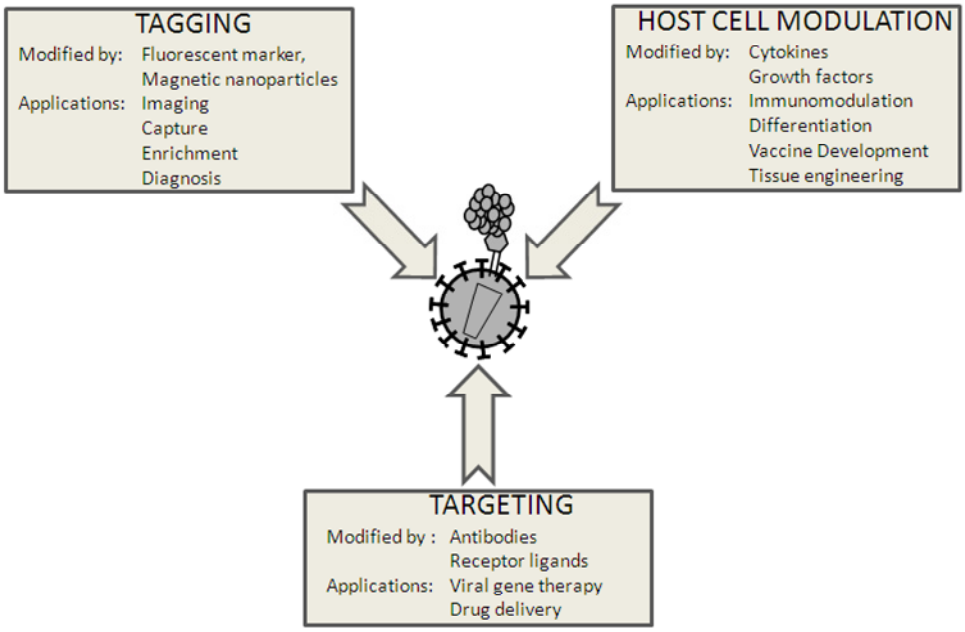

Figure 2: Possible applications for modifying viruses, VVs and VLPs with GPI-anchored proteins. Modification of viruses, VVs and VLPs using GPI-anchored proteins is useful for tagging of viruses, *e.g.* by using fluorescence markers for imaging or capture purposes. Modified viral surfaces can stimulate or inhibit host cell function, i.e. immune functions or induce differentiation. Modification also can re-direct binding specificities in order to target VVs or VLPs.

Virus Tagging

In its simplest application, GPI-anchored marker proteins may be used to identify and track viruses, VVs or VLPs. So far, viral particles are mainly labelled by incorporation of fusion proteins of viral structural proteins and (fluorescent) marker proteins [40,41]. However, these changes can also affect the biological behaviour of the particles. The use of GPI-anchored marker proteins provides an alternative, as the modification pathway does not interfere with viral structural components, i.e. the envelope surface protein required for target cell binding and infection. Furthermore, deposition of marker molecules on viral particles may be helpful for capture or enrichment prior to diagnostic procedures i.e. viral particles modified with 6 x his-tagged proteins can be enriched by using established immobilized metal ion affinity chromatography (IMAC) [42,43] or magnetic purification techniques [44]. This is especially interesting in cases where viruses tolerate concentration by centrifugation badly or where little is known about the virus, *e.g.* in the case of emergent viral diseases.

Modulation of Host Cell Functions

Interaction of virus particles with host molecules are often mediated by molecules located in the envelope. These interactions most importantly include immunological reactions. Modifying the envelope with immunologically competent molecules i.e. cytokines or growth factors allows for the manipulation of surrounding immune responses, thereby reducing possible side effects caused by systemic delivery of soluble agents [2]. This also includes protection of VVs from unwanted immune reactions such as complement activity [38]. Immunoprotection can help to ensure efficient delivery to target cells by eliminating premature inactivation of vectors in gene therapy approaches. Also presentation of antigen on the surface of VLPs is possible via the use of GPI-anchored molecules. These aspects can be of special importance in the development of vaccines or specific adjuvants enhancing vaccine efficacy. A further aspect of modulation of host cell function is inducing differentiation *e.g.* for tissue engineering purposes. Proof of principle was shown exemplary through differentiation of monocytes to dendritic cells [2].

Cellular Targeting

Viral glycoproteins located in the envelope function as recognition and entry devices to allow access to the target cells. Their specificity determines the host range or infection specificity of different virus species. Modification of these properties is crucial to achieve infection or trans-duction targeting – one of the most important goals of viral gene therapy. However, the direct manipulation of envelope glycoproteins i.e. by the generation of fusion proteins to redirect viral binding often leads to severely reduced infection efficiencies [45,46]. Deposition of specific binding factors by using GPI-anchored proteins may help to circumvent problems described previously, especially in cases where binding and entry of viral particles are mediated by independent factors [47,48]. Using viral painting technology under these circumstances has the advantage of flexibility. The same basic viral particle can be modified with a range of binding properties to suit the applications' specific needs. Antibody molecules, for example in form of single-chain antibody molecules, engineered to contain a GPI anchor, can provide a vast range of binding specificities. The same principle is applicable in drug delivery; however VVs and VLPs are less suitable for these approaches.

CELL SURFACE PAINTING WITH GPI-ANCHORED PROTEINS

Background

Cell surface engineering is a challenging but highly demanded task for biotechnological and medical applications. "Painting" a cell surface with a protein of choice is an attractive and powerful technology that can be achieved by decorating the plasma membrane with GPI-anchored proteins [33,49]. GPI-anchoring has the unique property that proteins with such a glypation moiety can stably incorporate into phospholipid bilayers of artificial planar membranes or cell membranes (Fig. **3**). This unusual ability of GPI-anchored proteins to re-insert into cell membranes was recognized in the framework of investigating functional aspects of the complement decay accelerating factor (DAF, CD55) to protect tissue from complement attacks. In a hallmark study, Medof and colleagues observed that the DAF protein preferentially re-incorporated into the surface of erythrocytes if the cells were incubated with purified membrane fractions of human red blood cells [50]. In fact, they described that purified DAF must have been re-inserted into the cell surface membrane, as DAF association with the erythrocyte's membrane could not be diminished by extensive washing of the cells even in the presence of high salt concentrations. However, the authors could extract membrane incorporated DAF with detergents. Moreover, they attributed some integral membrane properties to re-inserted DAF due to its functionality. This study is indeed remarkable as at that time the GPI-anchoring mechanism of DAF was not yet recognized, but followed three years later [51]. By elaborating on the incorporation phenomena of DAF, Medof and co-workers described that high- and low-density serum lipoproteins were able to interfere with the re-insertion [50]. In addition, membrane insertion of DAF occurred only at temperatures between 20 and 37°C, where a certain lateral mobility of lipids within the membrane is guaranteed.

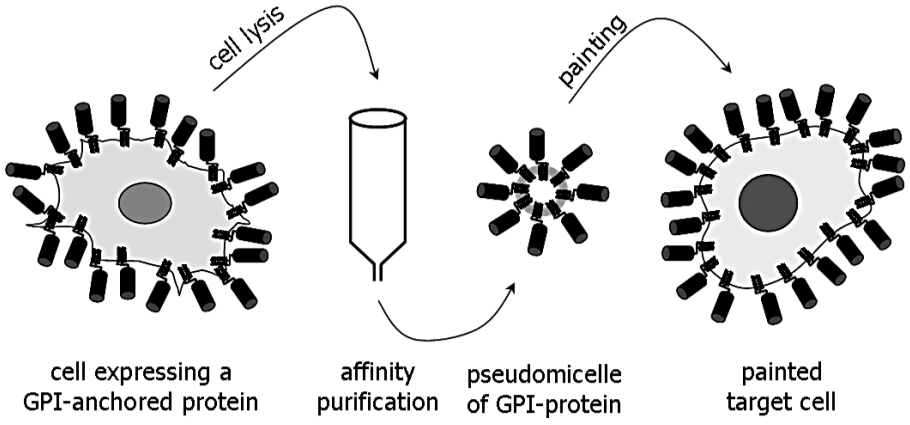

Figure 3: The principle of cell surface painting. Cells expressing an endogenous or recombinant GPI-anchored protein are lysed to generate a total protein extract which is used for affinity purification of the GPI-anchored protein. This can then be exogenously added to decorate the surface of a different cell or cell type.

State-of-the-Art

A pioneering study by Tykocinski and Medof then demonstrated that fusing the GPI anchor addition signal sequence (GSS) of DAF to the carboxyl terminus of the lymphocyte receptor CD8 permitted the expression of the fusion protein as GPI-anchored protein at the surface of transfected cells [37]. This study proved first evidences that

basically any protein can be expressed as GPI-anchored protein. Both natural and artificially generated GPI-anchored proteins can be purified to homogeneity and re-inserted into the outer leaflet of virtually any target cell of choice. In a review article, Medof and co-workers nicely worked out major advantages of this technology of cell surface engineering [33]: any proteins can be generated as GPI-anchored protein. GPI-anchored proteins can be inserted into the plasma membrane even of cells that are difficult to transfect or to transduce. Membrane insertion of GPI-anchored proteins is fast, efficient, and remarkably stable and does not require previous cell culturing. Multiple GPI-anchored proteins can be painted concurrently or sequentially on the same cell. The number of painted molecules can be easily varied depending on the incubation time and the protein concentration used. Finally and most importantly, cell surface painted GPI-anchored proteins retain their natural functions [33]. Two publications describe in depth methodological aspects of how to design, express, purify and functionally re-insert GPI-anchored proteins [34,52]. One peculiar property of GPI-anchored proteins is to participate in so called membrane microdomains or lipid rafts [53,54]. Membrane compartmentalization plays a key role in signal transduction events, as it dictates aggregation and segregation of proteins at the membrane. Today, still many aspects of the role of such membrane microdomains are not fully understood. Due to their preferential localization in membrane microdomains, GPI-anchored GFP molecules are used as fluorescent markers to investigate membrane microdomains and membrane microdomain-associated proteins [36,55-57]. However, it was noted that, most probably due to different lipid compositions of the GPI-anchor, particularly on existence or absence of a palmitoylation at the inositol ring, different GPI-anchors differentially localize in membrane microdomains [36,58]. Noteworthy, the most stringent membrane microdomain localization was attributed to DAF, or GFP-GPI(DAF) [36]. Moreover, transfected and painted GPI-anchored proteins (re-)localized identically efficient in membrane microdomains [36,59]. Interestingly, mixing carrier lipids that are predominantly found in membrane microdomains, such as cholesterol, sphingomyelin or dipalmitoyl-phosphatidylethanolamine [60], with purified recombinant GPI-anchored proteins increased the efficiency of cell surface painting, whereas non-raft lipids had no effect [36].

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PROTOCOL 6

Cell surface painting

1. *Wash target cells with pre-warmed PBS or serum-free medium by spinning cells down in a 15 ml tube. Certain GPI-anchored proteins have the tendency to stick to plastic, which may be avoided by using siliconized tubes.*
2. *Re-suspend cells in serum-free pre-warmed medium to a density of 10^6 to 10^7 cells per ml. Cells sensitive to serum-starvation may be painted in serum-containing medium with higher concentrations of GPI-anchored proteins.*
3. *Purified GPI-anchored proteins are added to a final concentration of 0.5 to 5 µg/ml.*
4. *Cells are incubated with purified GPI-anchored proteins at 37°C/5%CO_2 for 30 minutes to several hours. For many cell types cell surface painting reaches a plateau of insertion after 90 minutes of incubation. Cell sedimentation is prevented by occasional gentle mixing permitting efficient painting.*
5. *Extensively wash painted cells and verify painting efficiency by flow cytometry.*

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Biomedical Applications

The technique of cell surface painting is particularly interesting for biomedical applications. For example, erythrocytes that were painted with the adhesion molecule LFA-1 were then able to interact with target cells expressing its counter-receptor CD2 [61]. Decorating cells with an artificial high affinity ligand for the integrin αvβ3, termed KISS31, gained adhesive properties to interact with immobilized αvβ3 integrin *in vitro* [62]. As the integrin αvβ3 is up-regulated in tumour-associated blood vessels [63] targeting tumour-specific cytotoxic T cells to αvβ3 integrin-expressing tumours was proposed as strategy to fight against cancer. In fact, primary T cells painted with GPI-anchored KISS31 adhered to αvβ3 integrin *in vitro* and, if injected into tumour-bearing mice, homed to αvβ3 integrin-positive tumours *in vivo* [62]. Moreover, painting of target cells with peptide-loaded GPI-anchored MHC I complexes were efficiently recognized and killed by antigen-specific cytotoxic T cells *in vitro* [64]. Cell surface painting of GPI-anchored co-stimulatory molecules, such as CD80 and CD86, was shown to enhance cell proliferation [65]. Injection of murine tumour cell membranes, that were previously painted *in vitro* with GPI-anchored CD80, enhanced the expansion of tumour-specific cytotoxic T cells *in vivo* [66]. Furthermore, immunization with CD80-GPI-painted tumour cell membranes protected mice from a parental tumour challenge [66]. Further experiments are needed to exploit the painting strategy, particularly by re-inserting multiple GPI-anchored proteins in cells, for targeting tumours and other diseases *in vivo*. Cell painting may also be an alternative

to *ex vivo* gene therapy approaches, i.e. instead of transduction of therapeutic target cells, for example with retroviral vectors, the cells can be decorated with GPI-anchored proteins.

Beside above described efforts to enhance immunity, there is another interesting therapeutic potential of GPI-anchored proteins: Patients suffering from paroxysmal nocturnal hemoglobinuria bear a somatic mutation in the PIGA gene located on the X chromosome [67]. The enzyme PIGA is pivotal for the synthesis of the GPI moiety. In fact, patients with paroxysmal nocturnal hemoglobinuria show aberrant expression of the GPI-anchored protein DAF [68]. Consequently, erythrocytes from these patients are destroyed by complement-mediated lysis due to the lack of the complement-inhibitory function of DAF. Interestingly, cell-to-cell transfer of DAF was observed to occur *in vivo* in a transgenic mouse model [68]. This observation let to a clinical trial, where six patients suffering from paroxysmal nocturnal hemoglobinuria were transfused with erythrocytes derived from healthy donors [69]. At day one, three and seven after transfusion, DAF surface expression was detectable on recipient erythrocytes and granulocytes in all six patients [69], indicating that DAF derived from healthy cells were transferred simultaneously to deficient cells *in vivo*. This study suggests that simple transfusion and *in vivo* transfer of GPI-anchored DAF may have tremendous therapeutic potential to treat paroxysmal nocturnal hemoglobinuria patients.

SURFACE ENGINEERING OF MICRO- AND NANOPARTICLES WITH GPI-ANCHORED PROTEINS

Background

From the surface modification of cells, followed by enveloped viruses and viral vectors, the natural progression for GPI painting lies with micro- and nanoparticles, most obviously for those that are cell membrane derived or biomembrane-like. Giant unilamellar vesicles (GUVs) are in the ten to hundred micrometer range and can be produced by mixing diverse phospholipids and membrane components such as cholesterol and phosphatidylcholines under static electric current. They are widely used in membrane research due to their size and ease of handling for studying lipid phase separation and the appearance and behaviour of lipid raft domains [70]. Liposomes are also produced by mixing phospholipids in aqueous solution and, although varying in size, they are generally 2-3 dimensions smaller than GUVs. Liposomes have often been proposed as therapeutic agents as they are seemingly ideal in many ways [71]. They have an aqueous centre which can be used to carry drugs or therapeutic molecules (membrane bound if required), they can fuse with cells to deliver the therapeutic agent directly into or onto the target cell, they are non-toxic to biological systems and there are many modern biochemical possibilities for modifying their exterior surface to make them biocompatible, via PEGylation for example [72], or to target them (Fig. **4**). Other closely related, manmade particles of this dimension are micelles (the same as liposomes but with no aqueous centre) and nanoemulsions or various kinds of polymeric nanoparticles [71]. All such nanocarriers can have lipid bilayer or lipid bilayer-like properties and may have the potential for GPI modification.

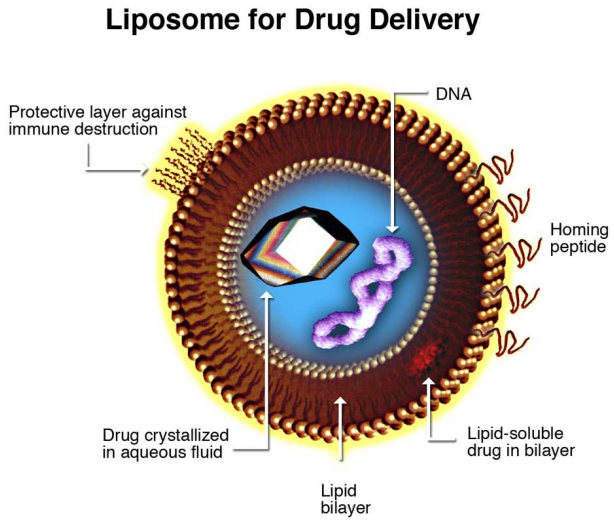

Figure 4: The concept of a multifunctional, liposome based nanocarrier as depicted by Kosi Gramatikoff [73]. Soluble drugs or therapeutic biomolecules (*e.g.* DNA, RNA, peptides, proteins, antibodies) can be incorporated into the aqueous centre and non-soluble drugs or molecules into the lipid bilayer. Immuno-protective and targeting agents can be incorporated chemically or potentially by means of GPI painting onto the surface.

Lipid bilayer membrane particles derived from mammalian cells, commonly known as membrane vesicles, typically lie in a size range of ten to low hundreds of nanometers. Exosomes, usually described as being in the 40 – 100 nm range, are generated by membrane invagination into multivesicular bodies within the cytoplasm and then released from the cell by exocytosis. Once outside, exosomes can also be referred to as membrane vesicles or micro-vesicles although such particles can also be formed by proteolytic cleavage of surface GPI-anchored proteins, apoptosis and mechanical stress to the cell membrane (and are as such a common component of laboratory cell culture systems) [74]. Generally, such vesicles have been widely studied in the field of cellular and molecular biology since the early 1960's but due to their composition, size and density it is worth to mention that they have often been confused with viruses and virus-like particles and *vice-versa*. Exosomes have been widely studied in their role for intercellular communication but, by containing microbial components, are also strongly linked with the cell-to-cell spread of infectious agents [75]. As they can be found in many easily accessible bodily fluids, such as blood and saliva, there is a great interest recently in their use as potential disease markers for diagnostics [76] and it is especially in this respect that a connection with GPI painting can be made.

Inorganic microparticles can also be surface engineered with GPI-anchored proteins, but in contrast to cells and viruses the GPI-anchor is not used, instead other chemistries available on the protein part of the GPI molecule, such as amino acid-based tags or individual amino acid modifications (allowing for example biotinylation), are be used for attachment. Although this is not painting in itself, this leaves the GPI anchor free for membrane attachment thereafter, adding an extra dimension to the painting system. Hence, correctly engineered GPI-anchored proteins may not only be painted onto to a membrane as a biofunctional unit but at the same time used as a linker to inorganic particles such as magnetic nanoparticles or quantum dots for many potential applications, i.e. biomembrane surfaces can be simultaneously functionalised not only with multiple active GPI-anchored proteins but also with diverse nanomaterials.

State of the Art

Synthetic Membrane Vesicles

Aside from the use of GUVs and liposomes as tools for the basic understanding of GPI-anchored protein biology, the use of GPI painting for modification of such particles can still be considered as an emerging area and published work remains sparse. However, there is a lot of current activity and results are starting to emerge. Bumgarner and co-workers have invented and characterised a method to engineer the surface of biodegradable microparticles with GPI-anchored proteins [77]. This stemmed from many earlier years of work from the same group of Selvaraj on cell painting and cancer vaccine development showing that most likely any protein can be engineered to have a GPI-anchor and stays functional as such [34,66,78-80]. In this recent study, albumin based microparticles (between 4.3 and 4.5 µm depending on the production technique), were painted with the immune regulatory proteins GPI-hB7-1 (or CD80) and GPI-mICAM-1 (or CD54) (Fig. **5**). The association between GPI-anchored protein and microparticle was characterised and optimised in terms of concentration, temperature and kinetics. As with cells and viruses, the association was shown to be reversible and dependent on the presence of an intact GPI-anchor. Although there is clearly an activity window, storage conditions over a week at 4°C were considered to be reasonable. Interestingly, both molecules could be simultaneously painted onto the same particles without any negative influence being seen, however, functionality of the molecules so far has only been demonstrated by means of antibody binding, so their true effectiveness in a medical setting still needs to be shown [77].

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PROTOCOL 7

Microparticle surface modification with GPI-anchored proteins:

1. *Suspend 1 mg of microparticles (this protocol is optimized for albumin microparticles) in 150 µl of PBS (pH 7.4) in a 1.5 ml microcentrifuge tube and incubate with 20 µg/ml of the GPI-anchored protein of interest for 20 mins at room temperature (the microcentrifuge tubes should be lightly tapped every 5 mins to insure good mixing).*
2. *Fill up the microcentrifuge tube with wash buffer (PBS/5 mM EDTA/1% FCS), centrifuge briefly to pellet the microparticles bearing the GPI-anchored protein and remove the supernatant by means of suction.*
3. *Repeat step 2 with 200 µl of wash buffer and then plate in triplicate 50 µl of microparticles in wash buffer into the wells of a 96-well V-bottom plate.*
4. *Add 50 µl of antibody directed against the GPI-anchored protein of interest to the wells (20 µg/ml) and shake the plate for 30 mins at 4°C on a plate shaker.*

5. *Wash the microparticles three times using wash buffer by filling the wells, centrifuging the plate to pellet the microparticles and removing the supernatant by suction.*
6. *After the 3rd wash, resuspend the pelleted microparticles in 50 µl of a suitable HRP-conjugated secondary antibody and incubate the plate for 30 mins at 4°C on a plate shaker.*
7. *Wash the microparticles two times with wash buffer by filling the wells, centrifuge the plate to pellet the microparticles and remove the supernatant by suction.*
8. *Resuspend the microparticles in PBS (pH 7.4) for the final wash, centrifuge the plate and resuspend the pelleted microparticles in 100 µl of peroxidase substrate TMB and observe for color development.*
9. *Stop the color development with 2N H_2SO_4 and centrifuge the V-bottomed plate at 1000 x g to pellet the microparticles.*
10. *Transfer 100 µl of the supernatant into a 96-well flat-bottomed ELISA plate and assay the color development at 415 nm using a microplate reader to verify binding of the GPI-anchored protein to the surface of the microparticle.*
11. *As a control for the reaction, incubate 1 mg of microparticles with 150 µl of buffer alone without the presence of the GPI-anchored protein.*

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Link to Nanotechnology

The last 5 to 10 years has seen dramatic increase of inter-disciplinary work to combine sub-micron materials such as diverse inorganic nanoparticles or carbon nanotubes with biological components such as nucleic acids, peptides, proteins or antibodies leading to the research area now known as bionanotechnology and there is much evidence to suggest that inventions from this area will play a big role in the future of medicine [82]. GPI-surface functionalisation is able to play a significant role here. The GPI-GFP-his and CD59his proteins used for painting of viral vectors were genetically modified to contain a 10 x histidine tag and this was specifically engineered into hydrophilic regions of the proteins which are regions protruding outwards from membrane binding region (35 and unpublished data). Histidine tags are able to bind metals such as gold, cobalt and nickel, whereby nickel nitrilo-tri-acetic acid (Ni-NTA) is the strongest due to its ion availability [83]. This was done not only to optimise the purification

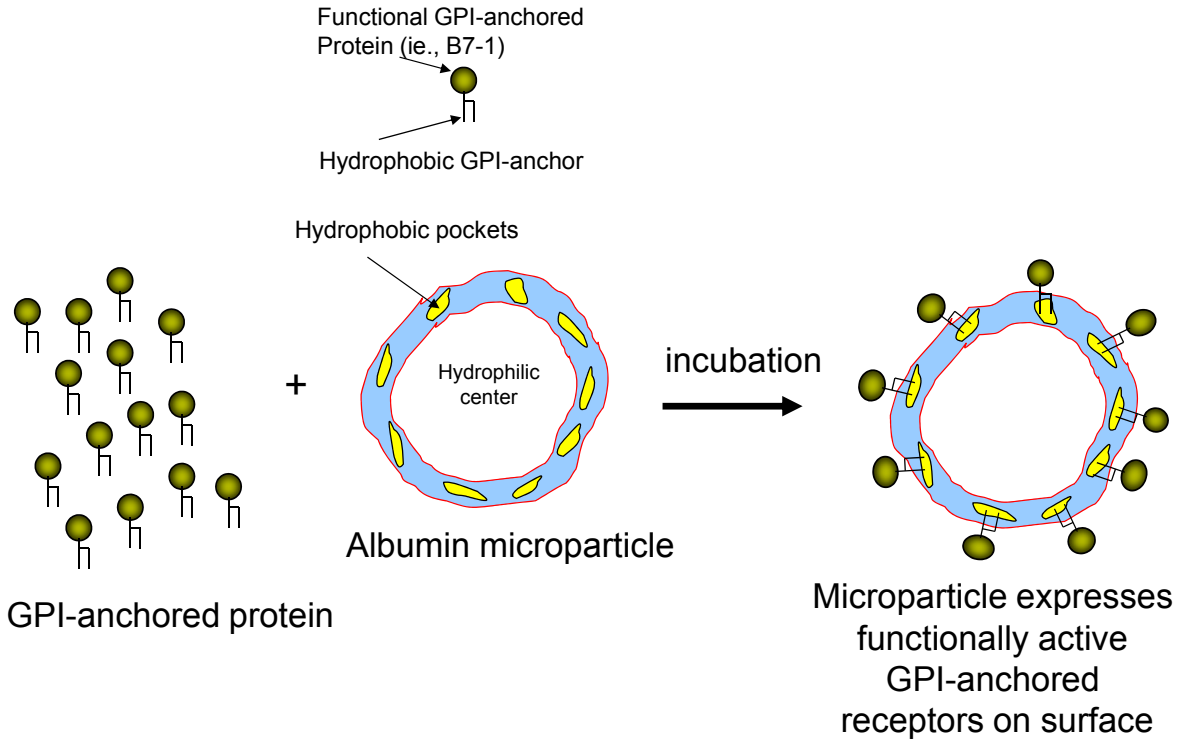

Figure 5: Schematic representation of the protein transfer process onto albumin microparticles [81].

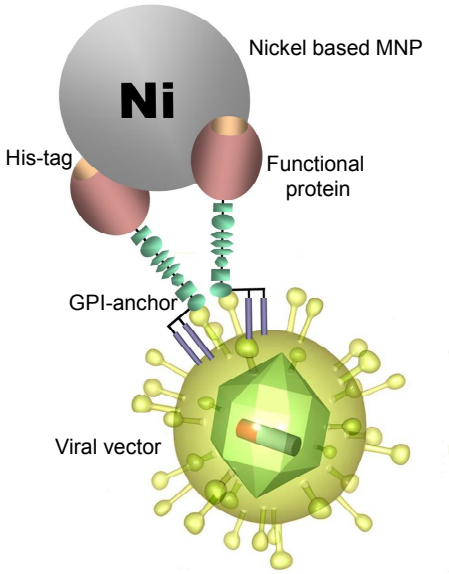

Figure 6: Linking organic and inorganic particles with GPI-anchored proteins. Proteins can be engineered to contain a GPI-anchor as well as a tag (*e.g.* his-tag) and remain functional. As such they can be used to link any membrane based entity such as a cell, virus, liposome etc (an enveloped retroviral vector is depicted here) to a nanomaterial designed to have a particular function for targeting, imaging etc (here a nickel based magnetic nanoparticle is shown).

procedure and alleviate the necessity for more expensive and timely antibody related methods, but also to allowspecific and orientation controllable linkage for the purified GPI-anchored proteins to virus-sized, nickel-based magnetic nanoparticles (Ni-MNPs). GPI-GFP-his and CD59his have been shown to bind several of such custom made Ni-MNPs, whereby iron core, Ni-NTA coated MNPs [84] were shown to have the highest binding efficiency and are also expected to be more biocompatible than pure nickel particles (unpublished data). Functionalised particles of this nature can then be painted onto the surface of viral vectors, cells, liposomes or membrane vesicles (Fig. **6**).

Potential Applications

GPI-Anchored Protein Production

When considering the use of GPI-anchored proteins for exogenous surface engineering, whether it be of cells, viruses or other particles, an important aspect is the production of the GPI-anchored protein itself. As bacteria do not make GPI-anchored proteins, expression systems are all based on eukaryotes. Currently, purification from mammalian cells is achieved by engineering in tags such as the histidine tag, by ion exchange chromatography or by using antibody related chro-matographical methods such as immuno-affinity or immuno-precipitation [35,36,62,77,85]. In bio-logical terms, the process of GPI anchoring was shown to be critically important by the fact that its abrogation leads to embryonic lethality [86], however, only around 1% of proteins are GPI-anchored in mammals. This is likely due, at least in part, to it being a costly and complicated process, requiring more than twenty gene products [87]. Therefore, yields of recombinant GPI-anchored proteins from mammalian expression systems are rather poor and purification can be technically tricky due to insolubility issues, i,e. the tendency to form micelles under aqueous conditions and in the absence of detergents. Despite their costs, there are some non-toxic detergents available for this (which are necessary for biological compatibility thereafter), but for future applications or off-the-shelf products this and the other obstacles described above will need to be overcome. One option is to optimise the production in mammalian cells. Although there is some evidence from work in insect cells that up-regulation of genetic factors within the GPI biosynthetic pathway may be an option [88], due to the multiplicity of factors involved, this approach may be limited and the most likely resort will be to up-scale the process. This is likely to be impractical and costly. One possibility is the completely synthetic production of GPI mimics or GPI-like molecules. Chemical biologists have already made significant progress here and this topic is addressed in Chapter 5. If one is to remain with biological approaches, expression in yeast systems may be interesting because of the far

easier possibility of process up-scaling. For cell-surface display purposes, it was seen that recombinant GPI-anchored proteins can be efficiently expressed in yeast [89,90]. Although there are some differences between mammalian and yeast GPIs, such as variations in the number of lipid prongs, recently it was found that human CD59his could be expressed in yeast (unpublished data), showing their potential as a GPI expression system per se.

Synthetic Membrane Vesicles

The interest for GPI surface modification of GUVs is likely to remain in the research area but for liposomes and other related nanocarriers, the key application clearly lies with the delivery of drugs or therapeutic molecules [71]. In this respect GPI modification may allow a simpler and quicker means (compared to chemical linkage which may also affect the functional activity of the molecule) to modify the surface of liposomes with proteins to target specific cells (*e.g.* receptor ligands), give immune-camouflage until they have reached their target (*e.g.* compliment regulatory molecules such as CD59) or link the liposome with nanomaterials for targeting and concentration at the therapeutic site (*e.g.* magnetic nanoparticles). Evidence already suggests that painting of albumin bioparticles has great potential for immune stimulation for vaccine approaches (*e.g.* adjuvant activity) and in turn enhance capacity as a targeted antigen or drug delivery device [81].

Bio-derived Membrane Microparticles

Membrane vesicles or micro-vesicles (MV) are interesting for GPI painting applications in a number of areas. MV can always be found in laboratory cell culture supernatants and several groups have activities in developing conditioned mediums as therapeutic agents. For example, evidence suggests that large complexes (100 – 220 nm) found in conditioned media from human mesenchymal stem cells are the agents responsible for significant cardioprotective effects in mouse and pig myocardial infarction models [91]. In the same way as discussed for liposomes above, MV may be functionalised with GPI-anchored molecules for targeting or immune-protection in such approaches. Many stem cell culture systems rely on the use of conditioned medium from so called feeder cells [92]. As it is highly likely that MV are an important component of such mediums, this opens the possibility for characterisation or enhancement of such mediums through GPI painting.

MV, in the form of exosomes, can be readily found in blood, urine, saliva, breast milk and a range of other bodily fluids and there is an ever increasing interest in their use as blood borne diagnostic markers for many diseases, including cancer [74-76,93-97]. Painting may have a diagnostics application in this respect as clinical samples could be painted with GPI tagged magnetic nanoparticles for quick and easy isolation and detection of specific MV disease markers. Similarly to what has been proposed for viruses, it may also be possible to introduce painted vesicles *in vivo* in order to track and image them for the basic understanding of the roles they play in the body or for monitoring disease progression.

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