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Review article

Maintaining sidedness and fluidity in cell membrane coatings supported on nano-particulate and planar surfaces

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ABSTRACT

Supported cell membrane coatings meet many requirements set to bioactive nanocarriers and materials, provided sidedness and fluidity of the natural membrane are maintained upon coating. However, the properties of a support-surface responsible for maintaining correct sidedness and fluidity are unknown. Here, we briefly review the properties of natural membranes and membrane-isolation methods, with focus on the asymmetric distribution of functional groups in natural membranes (sidedness) and the ability of molecules to float across a membrane to form functional domains (fluidity). This review concludes that hydrophilic sugar-residues of glycoproteins in the outer-leaflet of cell membranes direct the more hydrophobic inner-leaflet towards a support-surface to create a correctly-sided membrane coating, regardless of electrostatic double-layer interactions. On positively-charged support-surfaces however, strong, electrostatic double-layer attraction of negatively-charged membranes can impede homogeneous coating. In correctly-sided membrane coatings, fluidity is maintained regardless of whether the surface carries a positive or negative charge. However, membranes are frozen on positively-charged, highly-curved, small nanoparticles and localized nanoscopic structures on a support-surface. This leaves an unsupported membrane coating in between nanostructures on planar support-surfaces that is in dual-sided contact with its aqueous environment, yielding enhanced fluidity in membrane coatings on nano-structured, planar support-surfaces as compared with smooth ones.

1. Introduction

Cells are the basic structural and functional units of living organisms and have evolved with different biological functions and response mechanisms to environmental conditions. The cell membrane constitutes the interface between the cell and its environment. The cell membrane is relatively thin (4–10 nm) [1] and composed of a bilayer of lipids and proteins. Lipids are mainly phospholipids or sterols (generally cholesterol). Phospholipids and sterols both consist of fatty-acid-based lipids with a hydrophilic head-group and hydrophobic tail [2,3] that result in the spontaneous formation of a bi-layered cell membrane under physiological conditions (Fig. 1A). The cell membrane in essence constitutes a physical barrier with selective permeability for small molecules such as water and oxygen, whereas ions and higher molecular weight molecules, such as carbohydrates and antibiotics cannot directly pass through a cell membrane [3,4]. Moreover, the cell membrane is

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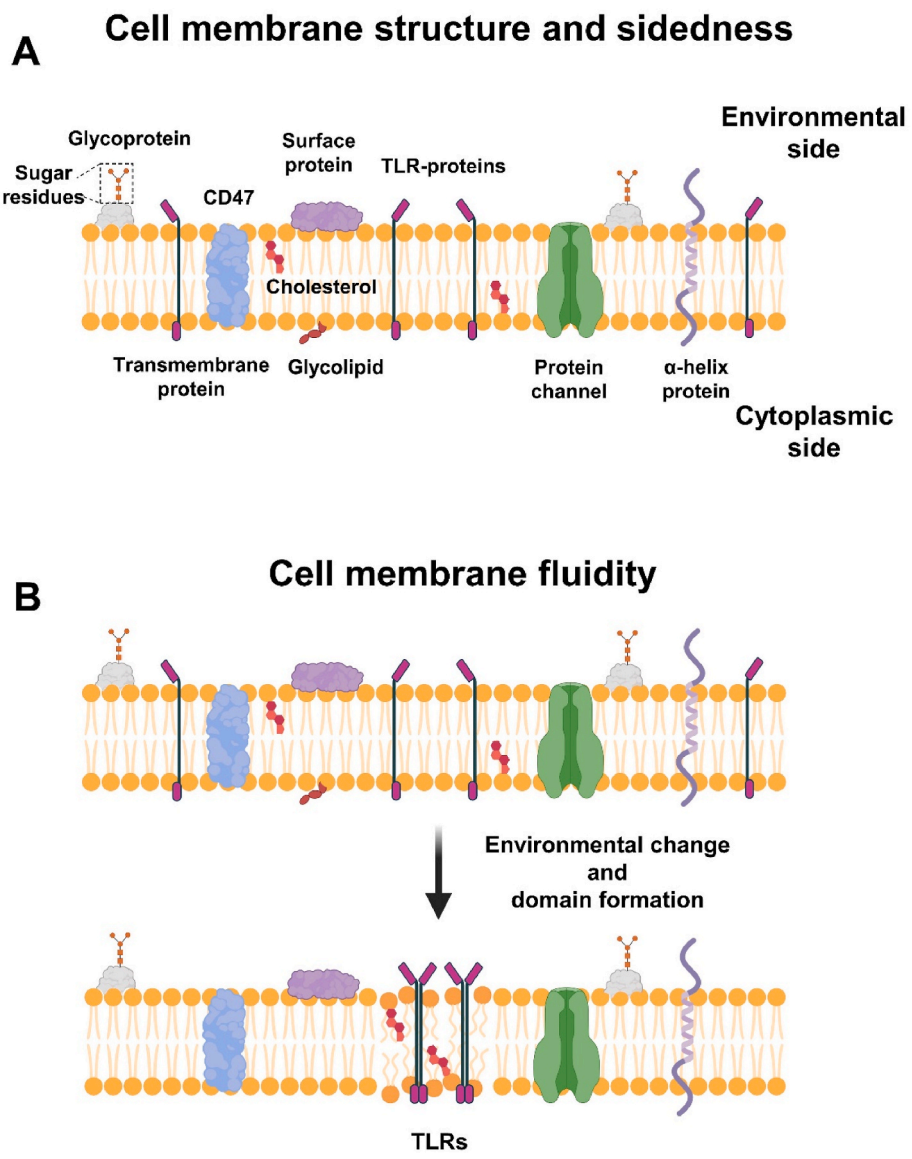


Fig. 1. **(A)** Cell membranes are asymmetric bilayers, made up of lipids and proteins. Functional components for interaction are located on the environmental and cytoplasmic side and possess different compositions on their environmental and cytoplasmic side. Preservation of the natural functions of cell membranes when applied in membrane coatings require maintaining of a correct sidedness, i.e. the right, environmental side-out upon coating by appropriate interaction with a nanoparticle or material surface [2,3]. **(B)** Adaptation of cell membrane coatings to environmental changes under physiological conditions requires maintaining of the dynamic organization of functional, membrane molecules existing in natural membranes (fluidity) [2,3]. Various proteins "float" around in small (100–200 nm) lipid rafts to create appropriate functional domains in the outer-leaflet, in response to environmental changes [26], including a wide variety of Toll-like receptors and other families of innate antigen receptors [29].

designed to be highly biocompatible, allowing prolonged circulation in blood and escape from immune cells [5,6]. Immune cell membranes also possess other unique functions that allow targeting to foreign bodies, carcinogenic tumor cells or invading pathogens and binding of pathogen-associated molecular patterns or cytokines [7,8].

This unique combination of different functionalities has stimulated an enormous interest in the potential use of cell membranes as a biomimetic coating of bioactive nanocarriers and materials exposed to the human body [9–11]. Differences between membranes of different cell types, methods to isolate cell membranes and the various potential applications of cell membrane coatings for tumor diagnosis and targeting, vaccine delivery, detoxification, pathogen clearance and control of immune diseases have been amply reviewed [12–16]. Despite the many reviews on cell membrane coatings, it is still unclear how to maintain the correct sidedness upon coating, i.e. the environmental side out, which is of crucial importance for the preservation of the natural functions of cell membranes (Fig. 1A) [2,3]. In a natural cell membrane under physiological conditions, the fundamental organizing features of the phospholipid bilayer are the polar head groups of phospholipids with uncharged or weakly negatively-charged groups in the outer-leaflet and strongly negatively charged groups in the inner-leaflet [17–22]. Glycoproteins with their hydrophilic sugar-residues pointing outwards on the outer-leaflet, play an important role in stabilizing the membrane structure [23].

Another unclear issue of crucial importance for the preservation of the natural functions of cell membrane coatings, still is how membrane fluidity is maintained in membrane coatings (Fig. 1B). Natural cell membranes are dynamic fluid structures in which lipids are, under physiological conditions, held together by a combination of Derjaguin-Landau-Verwey-Overbeek (DLVO) electrostatic double-layer interactions and attractive Lifshitz-Van der Waals forces [24,25], while various proteins float around in lipid-rafts to create appropriate

Table 1
Different cell types used for membrane isolation and coating, main functional properties of the different membranes and the responsible membrane components.

Cell type	Functional properties	Responsible membrane components
Red blood cells	Immune cell escape and long blood circulation	CD47, hydrophilic sugar residues [30,31,33,34].
	Adsorption of pore-forming toxin	Lipid bilayer [12,35].
Platelets	Immune cell escape and long blood circulation	CD47, hydrophilic sugar residues [31,36–38].
	Injury site targeting	Selectins and integrins [31,39,40].
Immune cells	Long blood circulation	CD47, hydrophilic sugar residues [41,42].
	Foreign body targeting	Antigen recognition receptors [43,44].
	Inflamed site targeting	Selectins and integrins [15,45–47].
	Cancer cell targeting	Antigen recognition receptors [16,47]
	Bacterial pathogen targeting	Antigen recognition receptors, Toll-like receptors [7,8,13].
	PAMP binding	Toll-like receptors [48,49].
	Cytokine binding	Cytokine receptors [46,48].
Tumor cells	Tumor cell adhesion and aggregation	Selectins and integrins [50,51]
	Modulation of immune cell response	Tumor antigens [14,52,53]
Stem cells	Immune cell escape	CD47, hydrophilic sugar residues [54,55]
	Inflamed or injury site targeting	Selectins and integrins [56–58]
Bacteria	Aggregation and co-adhesion	Ligands and receptors [59–61]
	Modulation of immune cell response	Ligands and receptors [62–64]

functional domains in the outer-leaflet in response to changes in environmental conditions [26]. Minor changes in membrane fluidity severely impact the biological function of the natural membrane, causing pathological processes [27,28].

In this review, a brief summary will be given of the structure, composition and functional properties of membranes of different cell types under physiological conditions and the impact of cell membrane isolation methods on the properties of resulting cell membranes harvested. Literature examples of the importance of correct sidedness and maintaining fluidity will be analyzed with the aim to answer the question which properties of a nano-particulate or planar material surface are responsible for maintaining correct sidedness and fluidity in a membrane coating.

2. Functional properties of membranes of different cell types

The summary in Table 1 of cell types used for membrane isolation and coating clearly indicates that there is a myriad of functional properties exhibited by cell membranes under physiological conditions. All membranes of blood-borne cells, including stem cells, facilitate immune cell escape (see also Table 1) and therewith long blood circulation. Blood circulation first of all requires a certain degree of hydrophilicity that is realized through the presence of hydrophilic sugar-residues of membrane glycoproteins on the outer-leaflet. Simultaneously, immune cell escape is realized through cluster of differentiation protein 47 (CD47) [30,31]. CD47 is a self-recognition, transmembrane protein composed of hydrophobic and hydrophilic, positively- and negatively-charged amino acid residues, of which the hydrophobic residues reside predominantly in the interior of the protein. When CD47 interacts with signal regulatory protein alpha (SIRPα), a signal regulatory pathway protein in immune cell membranes, it transmits inhibitory signals to the immune cell "not to eat" the cell displaying CD47. Nearly all targeting displayed by different cell types rely on antigen recognition receptors, of which selectins and integrins play a distinctly different role. Selectins play an important role in cell adhesion, although not involved in recognition. Integrins play an additional role in adhesion by interacting with extracellular matrix components. Toll-like receptors (TLR) constitute yet another type of immune cell receptors, composed of a variety of transmembrane proteins. A single TLR can be composed of three classes of main proteins forming a membrane domain, i.e. the receptor protein itself, a co-receptor protein enhancing ligand recognition and an adaptor protein to facilitate downstream signaling [32]. TLRs mainly expose themselves on a cell membrane or within endosomes and recognize a wide variety of pathogen-associated molecular patterns, initiating the production of pro-inflammatory cytokines and antimicrobial molecules. TLR4 for example, specifically binds lipopolysaccharide (LPS) from Gram-negative bacteria, while TLR2 binds to Gram-positive bacterial cell wall lipoteichoic acid and peptidoglycan. Also, physiologically-healthy cytokine levels are maintained by cytokine receptors on immune cells. Bacterial cells possess a myriad of ligand-receptor molecules on their surfaces that enable tissue cell adhesion and internalization, as well as aggregation and co-adhesion with other species of bacteria and fungi.

Table 2
Membrane isolation methods, mechanisms and their possible impact on isolated membranes.

Method	Mechanism	Features and membrane impact
Hypotonic-medium exposure	Osmotically-induced swelling and cell burst	- Does not require lysis buffer [30,72] - Low yield for nucleated cells [73]
Ultrasonication	Disruption of cell integrity	- High local membrane temperatures [49,67] - Membrane protein denaturation [68]
Homogenization	Mechanical cell disruption	- Requires purification and membrane fragment isolation [71,74]
Freeze-thawing	Cell disruption by rapidly formed ice crystals in the cytoplasm	- Ice-induced membrane protein unfolding [69,70] - Requires cryoprotectants [70,75]

Many synthetic coatings have been described that attempt to mimic the functional properties of natural cell membranes [9–11]. Hydrophilic poly(ethylene glycol)-b-poly(β-amino ester) (PEG-PAE) coatings for instance, can provide stealth properties to a nanocarrier, allowing prolonged circulation in blood and escape from immune cells, while the pH-responsiveness of PAE enables targeting of negatively charged bacterial pathogens in an infectious biofilm [65]. Synthetic liposomes have a similar lipid bi-layer structure as cell membranes. Synthetic liposomes can be loaded with both hydrophobic and hydrophilic drugs, fuse with target cells and when properly functionalized they can be stealthily transported in the blood circulation [66]. However, synthetic functional coatings lack the natural myriad of functional properties of natural liposomes and cell membranes, making cell membrane coatings attractive for many applications.

3. Cell membrane isolation methods

Cell membrane isolation methods (Table 2) should ensure preservation of the chemical composition, the integrity of the lipid bi-layer structure of the membrane and the functional properties of the membrane components. Amongst the cell membrane isolation methods listed in Table 2, ultrasonication is most frequently applied despite its possible denaturing impact on membrane proteins and therewith membrane functionality [49,67,68]. The ease of ultrasonication has to be balanced viz a viz preserving the functional properties of the membranes essential for the coating under development. Similar considerations must be made towards isolation using freeze-thawing that should not be applied when protein conformation is critical for the functionality of the membrane

Table 3
Direct and indirect methods for demonstrating correct sidedness of cell membrane coatings.

Method	Mechanism
DIRECT methods	
Particulate micro-electrophoresis [41,49]	Comparison of the zeta potentials of natural membranes or membrane fragments with zeta potentials of coatings
Fluorescent antibody-labelling [79,80]	Demonstration of the presence of selected outer- or inner-leaflet proteins
Sialidase assay [23,81]	Demonstration of the presence of hydrophilic, outer-leaflet sugar-residues (sialic acid)
INDIRECT methods	
Enzyme-activity assay [82–84]	Measurement of unique enzyme activity characteristic of an environmental or cytoplasmic membrane side
Assessment of functional behavior [41,75]	Demonstration of a wide variety of different functional properties of a coating viz a viz a corresponding natural membrane

coating aimed for [69,70].

Common to all methods listed, is the final step of collecting membrane fragments. High-speed centrifugation can be used to remove soluble proteins, cell nuclei and cytoplasm, but as a drawback, membrane fragments can aggregate, making them unsuitable for use as a membrane coating [30,69,71]. Membrane aggregation can be made undone by additional ultrasonication.

4. Cell membrane coating technologies

There are different technologies for coating of particulate and planar support surfaces, that include co-extrusion of isolated membrane fragments with particulate support materials [30], deposition of isolated membrane fragments on particulate [76] or planar [75] support surfaces in microfluidic devices, sonication of membrane fragments with nanoparticulate matter [49] or simple sedimentation of membrane fragments on a planar surface [77]. Confusingly, in the literature it is not always clear whether membrane coating is achieved using isolated membrane fragments or secreted outer membrane vesicles [62], that may or may not flatten like mammalian cells do upon contact with a substratum surface, depending on whether the surface is hydrophobic or hydrophilic [78]. *Stricto sensu* however, an outer membrane coating is not necessarily the same as a coating prepared using isolated membrane fragments and care should be taken in the interpretation of results. Although there is insufficient data available in the literature to allow firm conclusions about the role of coating technology in maintaining sidedness and fluidity, all technologies are generally assumed to yield correct sidedness sometimes after optimization of process parameters [12–14]. Maintenance of fluidity however, is less trivial for each of the technologies listed [15,16,30,33,41].

5. Demonstration of and maintaining correct sidedness in membrane coatings

Correct sidedness of membrane coatings can be demonstrated by a number of direct and indirect methods (Table 3). Direct methods aim to demonstrate physico-chemical or structural correspondences between the outer-leaflet of a membrane and membrane coatings, while indirect methods infer correct sidedness of membrane coatings from the preservation of functional properties of membrane coatings viz a viz the functional properties of natural membranes.

Polar head groups of phospholipids may be considered as the driving force for organizing membrane sidedness, that is stabilized by glycoproteins on the environmental side of a membrane [23]. Outer-leaflet glycoproteins are arranged with their sugar-residues pointing outwards, therewith creating a charge asymmetry across a membrane with a generally weakly negatively-charged outer-leaflet and strongly negatively-charged inner-leaflet [17–22]. This charge asymmetry of natural cell membranes can be used to decide on whether a membrane coating has correct sidedness or not by direct comparison of the zeta potentials of membrane fragments and natural membranes as compared with the zeta potential of membrane coatings. The zeta potential of membrane fragments as measured in suspension is comprised of the averages of the zeta potentials of the cytoplasmic and environmental sides of a membrane. Since the outer-leaflet has the weaker negative-charge, a correct sided membrane coating should have a zeta potential less negative than the membrane fragments applied for coating (Fig. 2A) [49].

On a more chemical basis, fluorescent antibody-labeling enables direct demonstration of the presence of selected outer- or inner-leaflet proteins on a membrane coating as an indication of correct sidedness. For instance, mitochondrial membranes possess anti-apoptosis protein Bcl-2 on their outer-leaflet, that could be demonstrated to be equally present in a mitochondrial membrane using fluorescent antibody-labeling as in a mitochondrial membrane coating on negatively-charged poly(lactic-co-glycolic acid) nanoparticles (Fig. 2B), from which correct sidedness was concluded [85]. Similarly, a sialidase assay can be carried out to verify a high content of hydrophilic sugar-residues in a membrane coating (Fig. 2C), as found on the outer-leaflet of natural cell membranes [23].

Ideally, cell membrane coatings replicate the multitude of cell membrane functionalities, amongst others through correct sidedness of the membrane. Membrane proteins possessing enzymatic functionalities are frequently spatially arranged on distinct sides of the membrane. Accordingly, correct sidedness can be indirectly inferred from the enzymatic functionality of a membrane coating viz a viz the functionality of the natural membrane. For example, ATPase is predominantly found in the inner-leaflet of mitochondrial membranes for the production of ATP. Correctly-sided membrane coatings should thus have a low ATPase activity (Fig. 2D) [84].

Preservation of multiple different functional properties of natural membranes in a membrane coating has recently also been suggested to indirectly indicate correct sidedness, although membrane fluidity plays a role here too. Bacterially-activated macrophage membrane coatings on nanowired, planar Si surfaces have been demonstrated to allow broad-spectrum adhesion in high numbers of all bacterial pathogens comprised in the so-called ESKAPE-panel of antibiotic-resistant strains (Fig. 2E) as well as adsorption of different pathogen-associated molecular patterns and cytokines [75]. Such a broad-spectrum demonstration of bacterial adhesion and molecular adsorption is considered unique to correctly-sided membrane coatings.

Zeta potential, hydrophobicity and structure are generally considered to play a key-role in maintaining correct sidedness of membrane coatings. RBC membrane coatings on negatively-charged, poly(lactic-co-glycolic acid) nanoparticles (zeta potential range −40 to −45 mV in PBS), had a correct sidedness over the diameter range from 65 to 340 nm, as demonstrated using a sialidase assay (Fig. 3A) [23]. Zeta potentials became more positive upon membrane coating, i.e. −25 mV similar as of RBCs, confirming correct sidedness. Interestingly, no uniform membrane coating could be obtained using positively-charged, PEI-coated nanoparticles with a zeta potential of +27 mV (Fig. 3B). A similar change towards more positive zeta potentials was reported for mitochondrial membranes on poly(lactic-co-glycolic acid) nanoparticles in PBS (150 mM), increasing from −42 mV towards −25 mV upon membrane coating [85]. Monocyte membranes attached to micelles with a diameter of 90 nm and a zeta potential of −25 mV in a phosphate buffer (10 mM), possessed a correct sidedness, as concluded from a zeta potential comparison with the one of membrane fragments (−47 mV) [49].

Thus, nanoparticulate and planar materials surfaces must be negatively-charged in order to attract a homogeneous, correctly-sided membrane coating. However, nanostructuring of a planar support surface reduces the interaction of membranes with the surface greatly [75,

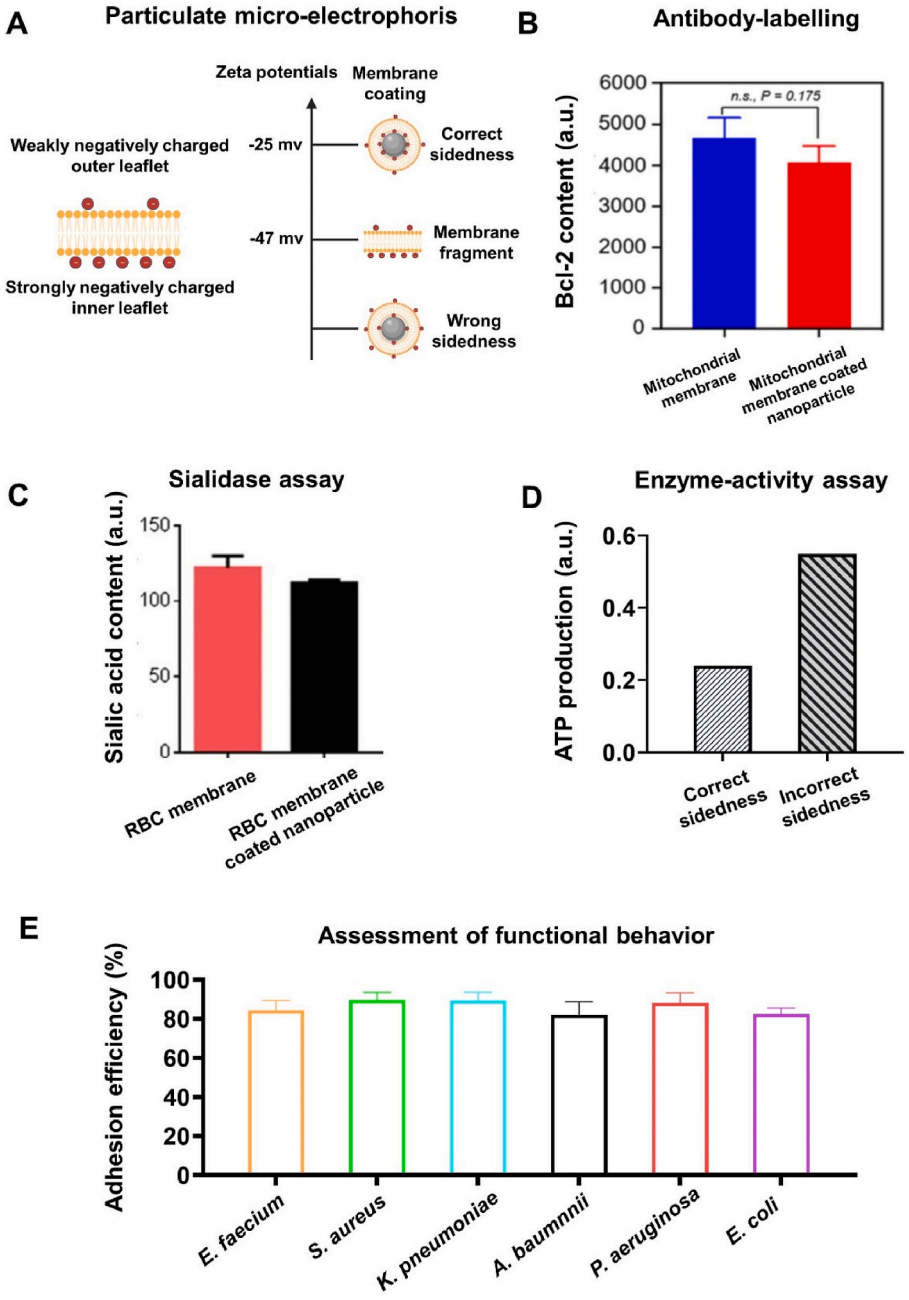


Fig. 2. Selected methods to demonstrate correct sidedness of membrane coatings. (A) Comparison of the zeta potentials of membrane fragments and a membrane coating demonstrates correct sidedness, since fragments present an average of the outer- and inner-leaflet zeta potential while the outer-leaflet possesses a weaker negative charge than the inner-leaflet [17–22]. Accordingly, a correctly-sided membrane coating should have a less negative zeta potentials than membrane fragments. Data were obtained in a phosphate buffer (10 mM), as taken from Ref. [49]. **(B)** Comparison of the fluorescence intensity of antibody-labeled mitochondrial membranes and mitochondrial membrane coated, poly(lactic-co-glycolic acid) nanoparticles. A correctly-sided membrane coating will present a similar fluorescence intensity due to fluorescently-labeled anti-apoptosis protein Bcl-2 as the mitochondrial membrane. Data were obtained in phosphate-buffered saline (PBS; 150 mM) [85]. Copyright 2021, ACS Publications. **(C)** Comparison of the sialic acid content of an RBC membrane and RBC membrane coated PLGA nanoparticles to identify the membrane sidedness. Sialic acid is mainly distributed on the extracellular surface of the RBC, a relatively high content of sialic acid on the membrane coating presents correct sidedness. Data were obtained in PBS (150 mM) [23]. Copyright 2014, Royal Society of Chemistry. **(D)** Comparison of the ATPase activity of mitochondrial outer membrane vesicles with a correct and incorrect sidedness. Vesicles with an incorrect sidedness were artificially made by repeated freeze-thawing. Correct sidedness of membrane coatings can be concluded from a low ATP production. Data were obtained in 3-(N-Morpholino) propanesulfonic acid buffer (10 mM) with sucrose (330 mM) added, as taken from Ref. [84]. **(E)** Broad-spectrum adhesion of different bacterial strains comprised in the ESKAPE panel of antibiotic-resistant pathogens on a bacterially-activated macrophage membrane coating on a nanowired, planar Si support surface. Data were obtained in PBS (150 mM), as taken from Ref. [75].

86]. Whereas positively-charged polyethylenimine (PEI)-coated nanoparticles with diameters up to 340 nm could not be uniformly coated with an RBC membrane (Fig. 3B), tenfold larger nanoporous Si particles (diameter of 3.2 μm) modified with (3-aminopropyl)triethoxysilane (zeta potential +7 mV in PBS (150 mM)) could be coated with a correctly-sided leukocyte membrane [41]. Correct sidedness was concluded from zeta potential comparison, sialic acid demonstration and multiple, functional properties including immune cell escape,

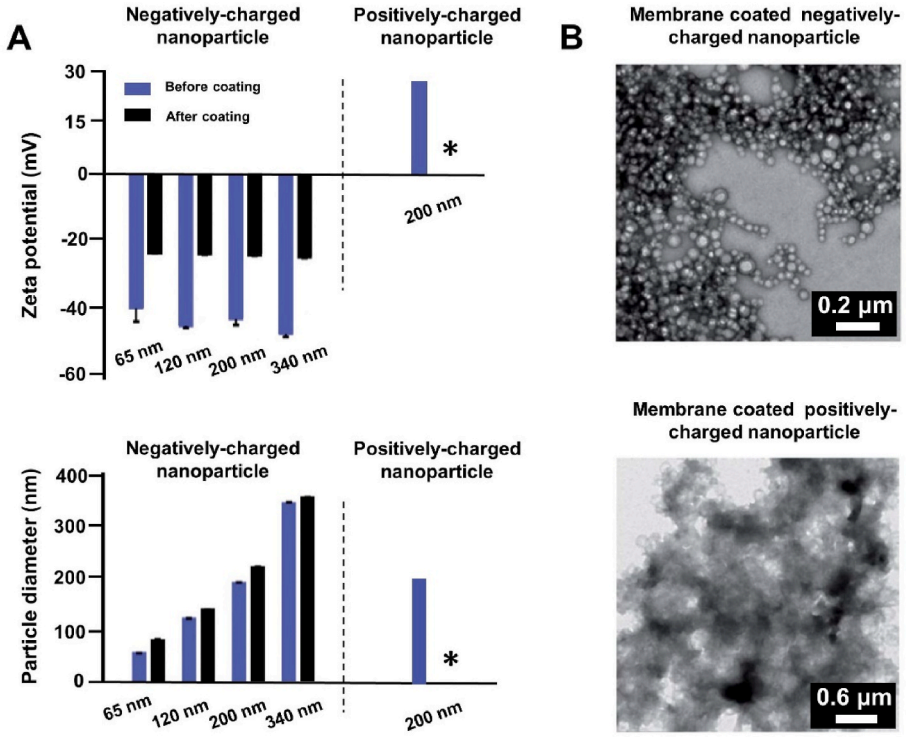


Fig. 3. Selected studies relating correct sidedness of membrane coatings with specific properties of the support surface and proposed mechanism of maintaining sidedness. (A) Diameters and zeta potentials in PBS (150 mM) of positively-charged, PEI-coated and negatively-charged, native poly(lactic-co-glycolic acid) nanoparticles before and after coating with RBC membranes. Note no homogeneous membrane coating could be applied on a positively-charged nanoparticle and hence zeta potentials and diameters could not be measured (see asterisks). Data taken from Ref. [23]. **(B)** SEM micrographs of a homogeneous RBC membrane coating on negative-charged, native poly(lactic-co-glycolic acid) nanoparticles and aggregation of membranes on positively-charged PEI-coated ones [23]. Copyright 2014, Royal Society of Chemistry.

enhanced blood circulation time and tumor cell targeting. Positively-charged, (3-aminopropyl)triethoxysilane (APTES) modified, nanowired planar Si surfaces (zeta potential +15 mV) in PBS (150 mM) could be coated with bacterially-activated macrophage membranes with a correct sidedness, as concluded from the facts that all members of the ESKAPE panel of antibiotic-resistant pathogens adhered to such membrane coatings and a large variety of pathogen-associated molecular patterns and cytokines adsorbed to them [75]. Interestingly, these functional properties of macrophage membranes were much less prominent when membrane coatings were applied to smooth, planar APTES-modified Si surfaces.

Collectively, it is concluded that correct sidedness is generally observed on negatively-charged, smooth support surfaces, while positively-charged support surfaces can be difficult to homogeneously coat due to overly strong electrostatic double-layer attraction between negatively-charged membrane fragments and the positively-charged support surface. However, positively-charged, nanoporous nanoparticles or nanowired, planar surfaces possessing curved structures can be homogeneously membrane coated with a correct sidedness because electrostatic double-layer attraction is confined to nanoscopic structures on the support surface. Taken together, these findings imply that correct sidedness does not result from electrostatic double-layer interactions between a membrane and a support surface. Moreover, electrostatic double-layer interactions between membrane fragments and a support surface are relatively small in high ionic strength suspensions and are dominated by attractive Lifshitz-Van der Waals forces at close approach. Instead, sidedness is directed by the hydrophilicity of the outer-leaflet possessing hydrophilic sugar-residues that are absent in the inner-leaflet. Correct sidedness is thus a result of strong, attractive Lifshitz-Van Waals forces between the inner-leaflet components and a support surface and between outer-leaflet sugar-residues and the aqueous environment in which coating takes place.

Table 4
Direct and indirect methods for demonstrating fluidity in cell membrane coatings.

Method	Mechanism	References
DIRECT methods		
Fluorescence recovery after photobleaching	Observation of fluorescence recovery of photobleached, fluorescently labeled membrane area	[77]
Fluorescence anisotropy	Monitoring rotation of a fluorescent probe in a labeled membrane	[87]
Laurdan generalized polarization	Measurement of the ratio of blue and red fluorescence intensity of Laurdan incorporated in a membrane	[88,89]
INDIRECT methods		
Assessment of functional behavior	Demonstration of a wide variety of different functional properties of a coating viz a viz a corresponding natural membrane	[75]

6. Demonstration and maintaining of fluidity in membrane coatings

Fluidity of membrane coatings can be demonstrated by a number of direct and indirect methods (Table 4).

Most direct methods to demonstrate fluidity in membrane coatings are based on monitoring the lateral diffusion of a fluorescent molecule or nanoparticle in a coating. By selectively photobleaching a specific region in a fluorescently labeled membrane coating, and subsequently observing the recovery of fluorescence intensity due to the lateral diffusion of labeled molecules from the surrounding areas, fluidity can

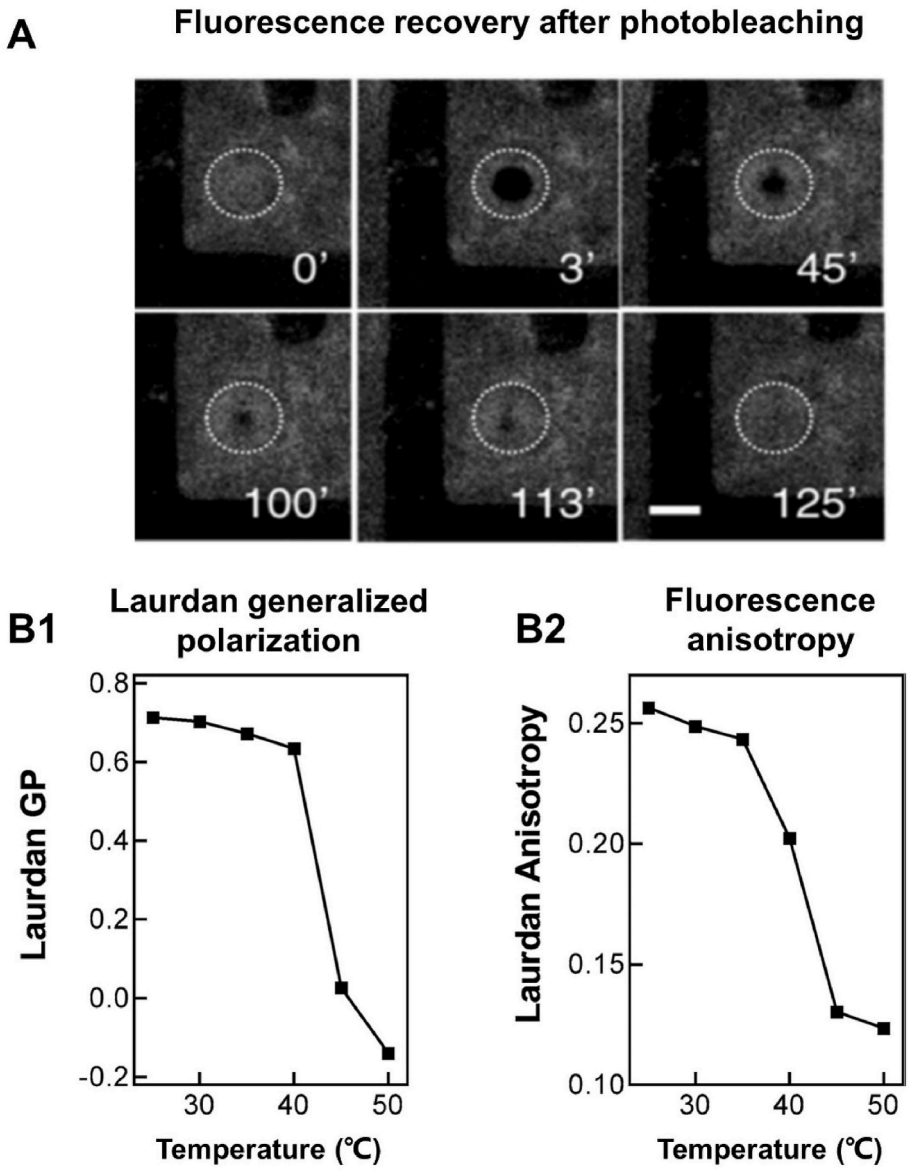


Fig. 4. Selected methods to demonstrate fluidity in membrane coatings. (A) Fluorescence recovery after photobleaching of RBC membrane coatings on a tubular carbon support surface for detection of hemolytic toxins in PBS (150 mM). Membrane coatings were labeled with red-fluorescent DiD, staining hydrophobic membrane components and fluorescence intensity recovery of a photobleached region imaged over time [77]. Copyright 2019, ACS Publications. **(B)** Laurdan generalized polarization (GP, panel **B1**) and simultaneously measured fluorescence anisotropy (panel **B2**) in DPPC, artificial membranes in sodium citrate/citric acid buffer (10 mM) with KCl (160 mM) added as a function of temperature, demonstrating membrane fluidity can be "frozen" at temperatures below 40 °C. Emission intensity was measured at 435 and 500 nm upon excitation at 350 nm [89]. Copyright 2002, Elsevier.

be directly demonstrated (Fig. 4A) [77]. Laurdan is a special fluorescent molecule that exhibits a red-shifted fluorescence in a less polar environment and a blue-shifted fluorescence in a more polar environment. This is expressed in the so-called generalized polarization ratio (GP) according to Parasassi et al. [90].

$$GP = \frac{I_{435} - I_{500}}{I_{435} + I_{500}}$$

in which I_{435} and I_{500} represent the blue and red-fluorescence intensities, respectively. Higher, more positive ratios indicate a more rigid lipid environment and lower or negative ratios are indicative of fluidity (Fig. 4B1) [89]. Fluorescence anisotropy is based on the rotational movement of fluorescent molecules in a membrane and fluorescence emission by polarized light, yielding emission of polarized fluorescence. Anisotropy is quantitated by the ratio of the parallel and perpendicular emitted fluorescence intensities and ranges from 0 (complete rotational freedom) corresponding with membrane fluidity to 1 (complete restriction of rotational freedom; Fig. 4B2) [89].

Similar as with the demonstration of sidedness, indirect demonstration of fluidity based on exhibition of a wide variety of different functional properties can be taken as an indication of fluidity in membrane coatings. However, at this stage it is important to note that in general both correct sidedness and fluidity are required for maintaining a wide variety of functional properties. Caution must thus be exercised in drawing conclusions about fluidity and sidedness separately when based on functional properties.

Once a correctly-sided membrane coating has been obtained, zeta potentials play a minor role in maintaining fluidity in membrane coatings, particularly under physiological conditions, characterized by a relatively high pH (7.4) and ionic strength (around 150 mM). Fluorescence recovery was absent, demonstrating frozen membrane coatings of egg phosphatidylcholine under high ionic strength conditions supported

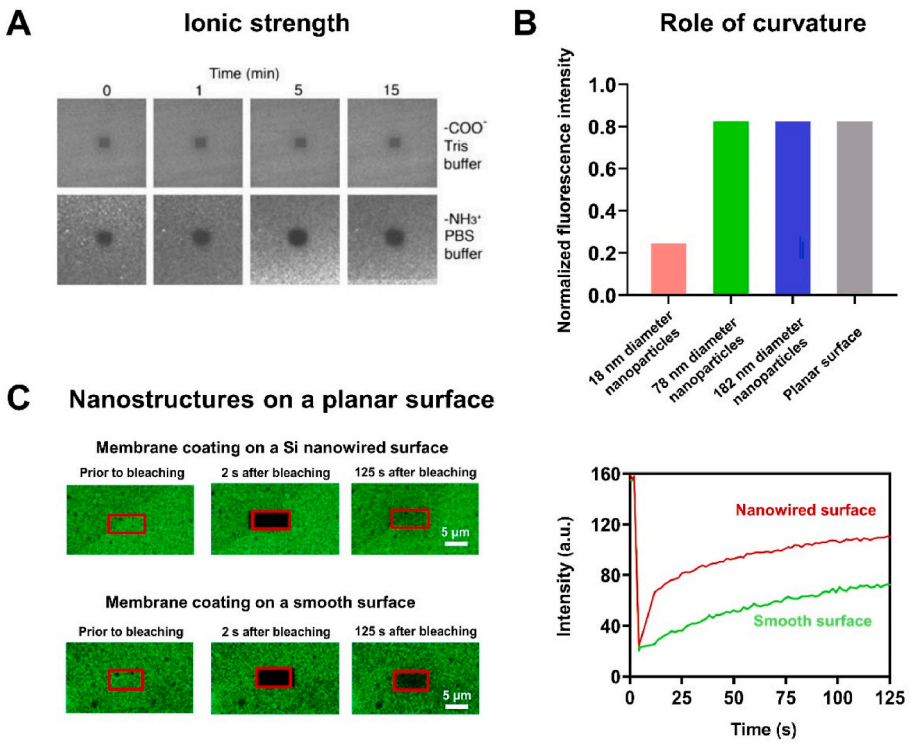


Fig. 5. Selected studies relating fluidity in membrane coatings with specific properties of the support surface and proposed mechanism of maintaining fluidity. (A) Fluorescence images at different times after photobleaching of egg phosphatidylcholine membranes adsorbed on -COO^- or –NH_3^+ terminated thiols in self-assembled monolayers on planar Au surfaces, measured in PBS (150 mM) and tris(hydroxymethyl)aminomethane buffer (150 mM), respectively. Fluorescence labeling was done with tetramethyl-rhodamine [91]. Copyright 2006, Cell Press. **(B)** Fluorescence recovery of DOPC lipid bi-layers supported on nano-particulate and planar, negatively-charged SiO_2 surfaces, measured 20 s after photobleaching in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (10 mM). Membranes were made fluorescent using tetramethyl-rhodamine. Data taken from Ref. [25]. **(C)** Fluorescence images at different times after photobleaching of bacterially-activated macrophage membrane coatings on positively-charged, APTES-modified smooth and nanowired planar Si support surfaces, measured in PBS (150 mM). Fluorescence labeling was done with 3,3′-dioctadecyloxacarbocyanine perchlorate [75]. Copyright 2021, Wiley-VCH GmbH.

on negatively-charged as well as on positively-charged, self-assembling monolayers on planar Au surfaces of 10-carboxy-1-decanethiol and 11-aminino-1-undecanethiol (Fig. 5A) [91]. Dioleoyl phosphatidylcholine (DOPC) lipid membranes supported on a smooth, planar and negatively-charged SiO_2 nano-particulate surfaces maintained fluidity when nanoparticles had diameters above 78 nm, while membranes were frozen on highly-curved nanoparticles with a diameter of 18 nm (Fig. 5B) [25]. Bacterially-activated macrophage membrane coatings on smooth, positively-charged APTES-modified, planar Si surfaces demonstrated limited fluidity, but oppositely had a high fluidity on positively-charged, APTES-modified, nanowired, planar Si surfaces, possessing highly-curved-structures (Fig. 5C).

Collectively, it is concluded that in case of large diameter nanoparticles (low curvature) and smooth, planar support surfaces with a correctly-sided membrane coating, fluidity is maintained, independent of whether the surfaces coated carry a positive or negative charge. However, membranes are frozen on highly-curved, positively-charged, small nanoparticles as high curvature requires the lipid bi-layer of the membrane to bend severely. This expands the distance between lipid molecules in order to accommodate membrane components to the curved support and therewith the force-balance in a membrane is affected which locally freezes the membrane. However, positively-charged, nanoscopic structures on a planar support surface strongly attach negatively-charged cell membranes yielding localized, frozen areas. The strongly attached, localized, frozen areas span the membrane over a nanostructured, planar support surface, leaving the majority of the membrane coating in dual-sided contact with an aqueous environment. This explains the enhanced fluidity of membrane coatings on nanostructured, planar surfaces as compared with smooth, planar ones. Accordingly, it can be expected that the density and height of nanoscopic structures on a support surface will also be influential on the resulting fluidity in membrane coatings.

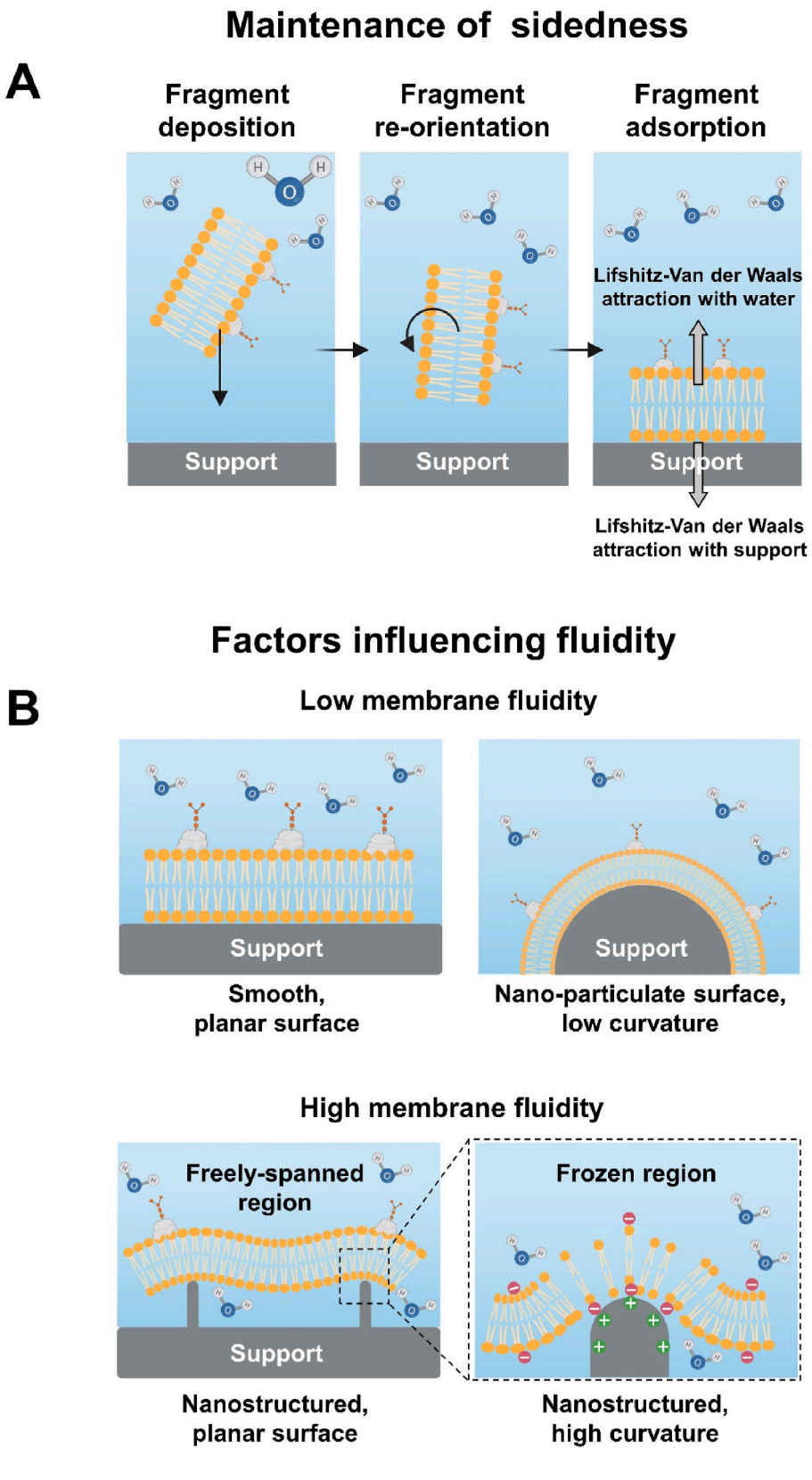
Table 5
Role of surface properties on maintaining sidedness and fluidity in supported membrane coatings.

Surface type	Negatively-charged surface	Positively-charged surface
SIDEDNESS		
Smooth	Correct sidedness, directed by outer-leaflet hydrophilicity (or inner-leaflet hydrophobicity)	Homogeneous coatings may be difficult to obtain
Nanostructured	No literature found	Correct sidedness, directed by outer-leaflet hydrophilicity (or inner-leaflet hydrophobicity)
FLUIDITY		
Smooth	Fluidity (frozen on small nanoparticles)	Limited fluidity
Nanostructured	No literature found	Frozen membranes on highly-curved curved nanostructures High fluidity on nanostructured, planar support surfaces

7. Conclusions

Table 5 presents a summary of the role of the properties of nanoparticulate and planar support surfaces in maintaining sidedness and fluidity in membrane coatings.

Zeta potentials, as an expression of surface charge under specific



(caption on next page)

Fig. 6. Maintaining correct sidedness and fluidity in supported membrane coatings. (A) During membrane coating, membrane fragments randomly oriented in suspension, deposit on a support surface. During approach, membrane fragments re-orient themselves with their hydrophilic sugar-residues in the outer-leaflet towards the aqueous environment and the more hydrophobic inner-leaflet towards the support surface. Electrostatic double-layer interactions between membrane fragments and a support surface are relatively small in high ionic strength suspensions and dominated by attractive Lifshitz-Van der Waals forces at close approach (below around 3 nm) according to the DLVO-theory of colloidal stability. Accordingly, correct sidedness is established by attractive Lifshitz-Van der Waals forces between the outer-leaflet and water and the inner-leaflet and the support surfaces. **(B)** Membrane fluidity is maintained on smooth, planar support surfaces and nano-particulate materials with a relatively large radius of curvature (low curvature). Nano-particulate materials with a small radius of curvature (high curvature) require severe bending of the membrane to accommodate adsorption requiring attractive electro-static double-layer attraction in addition to Lifshitz-Van der Waals attraction, yielding expansion of the membrane and affecting the force balance in the membrane, which yields a frozen membrane, i.e. restricted fluidity. Positively-charged nanostructures on a planar support surface form anchoring points attaching locally frozen membrane in between which are freely spanned and dual-sided surrounded by water yielding enhanced membrane fluidity as compared with the fluidity observed on a positively-charged smooth, planar support surface.

ionic strength conditions, appeared not to be a dominant factor in maintaining sidedness. We conclude that sidedness is directed by the presence of hydrophilic sugar-residues in the outer-leaflet of cell membranes (Fig. 6A) and that are absent in the inner-leaflet (see Fig. 1) [22,23]. This also explains why in general correct-sidedness is obtained regardless of the coating technology applied.

Accordingly, in all coating technologies, the outer-leaflet is directed to the aqueous environment of the membrane by attractive Lifshitz-Van der Waals forces between the sugar-residues and water molecules. Similarly, the inner-leaflet is directed to the surface to be coated by attractive Lifshitz-Van der Waals forces between inner-leaflet components and a support surface. Electrostatic double-layer repulsion between membranes and negatively-charged support surfaces is little influential especially in high ionic strength conditions, because at close approach Lifshitz-Van der Waals attraction become stronger than electrostatic double-layer repulsion [24,92]. On positively-charged surfaces however, absence of electrostatic double-layer repulsion and Lifshitz-Van der Waals attraction may lead to an inhomogeneous coating [23].

Fluidity in a correctly-sided membrane coating is generally well maintained regardless of the surface charge of relatively planar support surface by the balance between electrostatic double-layer interaction and Lifshitz-Van der Waals attraction. This balance however, can be critically affected by parameters of the coating process that may require optimization. The balance of electrostatic double-layer and Lifshitz-Van der Waals attraction between membranes on positively-charged nanoscopic support structures on the other hand, may yield a frozen membrane [25]. Although membrane-freezing should be avoided in the coating of nanoparticles, it endows enhanced fluidity in a membrane coating on nanostructured, planar surfaces as compared with planar surfaces. On nanostructured planar surfaces, the membrane is strongly attached and frozen to highly localized nanostructures (Fig. 6B) [75]. The majority of the membrane however, is freely spanned between nanoscopic, localized attachment points and in dual-sided contact with its aqueous environment that stimulates fluidity, similar as under physiological conditions.

8. Outlook and challenges

The conclusions of this review provide a guideline for future use of the unique properties of cell membranes in membrane coatings on nanoparticulate and planar materials surfaces with various applications in nanomedicine. Specifically, it guides the field in making the proper choices with respect to materials to be used as a support surface. Taken together, particularly with respect to maintaining fluidity, conclusions point to the use of nanostructured support surfaces. However, nanostructured surfaces can present themselves with a myriad of different nanoscaled morphologies [93]. A future challenge worthwhile to pursue would be to perform a detailed comparison of membrane coating technologies and their impact on maintaining sidedness and fluidity identify nanoscale structured supports that optimally maintain correct sidedness and particularly fluidity in membrane coatings.

Contributions

H.J.B., H.C.M., J.L., J.D.L., L.S and Y.R. conceived the study and supervised the project. S.L. and Y.L. searched and collected data from references. All authors interpreted the results. S.L. and Y.L. wrote the draft of the paper. H.J.B., H.C.M., J.L., J.D.L., L.S and Y.R. revised the manuscript. All authors critically reviewed the manuscript.

Declaration of competing interest

HJB is also director of a consulting company, SASA BV (GN Schutterlaan 4, 9797 PC Thesinge, The Netherlands). The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article.

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