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Chromatographic and electrophoretic methods for nanodisc purification and analysis

Abstract: Soluble nanoscale lipid bilayers, termed nanodiscs, are widely used in science for studying the membrane-anchored and integral membrane protein complexes under defined experimental conditions. Although their formation occurs by a self-assembly process, nanodisc purification and the verification of proper reconstitution are still major challenges during the sample preparation. This review gives an overview of the methods used for purifying and analyzing nanodiscs and nanodisc-reconstituted membrane proteins, with an emphasis on the chromatographic and electrophoretic approaches.

Keywords: chromatography; free flow electrophoresis; mass spectrometry; nanodiscs; native PAGE.

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Introduction

Soluble nanoscale lipid bilayers, termed nanodiscs (Bayburt and Sligar 2003), are excellent biomimetic systems for studying the membrane-anchored and integral membrane protein complexes under defined experimental conditions. Assembled from membrane scaffold proteins (MSPs), a nanodisc consists of two MSPs encircling a planar lipid bilayer in a double-belt configuration (Figure 1). The amphiphilic helical structure of the MSPs shields the hydrophobic edge of the lipid bilayer and stabilizes discrete disc sizes determined by the length of the MSPs. The advantages of using this system include water solubility, narrow size distribution, flexible lipid composition, access to both sides of the bilayer simultaneously, minimal light scattering, faster diffusion, stability in shear flow (Bayburt and Sligar 2010), and controlled stoichiometry of the disc to the target protein. Despite still being a relative novel approach for membrane protein study, several examples of membrane proteins reconstituted into nanodiscs exist, for example, G-protein coupled receptors, cytochrome P450s, and ATP-driven pumps (Supplementary Table 1). Nanodisc-reconstituted membrane proteins can essentially be handled as soluble proteins in aqueous solution. This facilitates the application of analytical techniques that are normally difficult to use in the study of membrane proteins such as surface plasmon resonance, nuclear magnetic resonance (NMR) spectroscopy, and electron paramagnetic resonance spectroscopy.

Although their formation occurs by a self-assembly process, nanodisc purification and the verification of proper reconstitution are still major challenges during the sample preparation, and often, a multitude of techniques are used in combination to obtain well-defined preparations. Only recently, a progress in native mass spectrometry might allow in the near future a mass spectral analysis of intact nanodisc complexes at single lipid resolution (Hopper et al. 2013, Marty et al. 2013, 2014). Several excellent reviews have surveyed methods used for analyzing and purifying nanodiscs and nanodisc-reconstituted membrane proteins (Nath et al. 2007, Ritchie et al. 2009a, Bayburt and Sligar 2010, Inagaki et al. 2013). Here, we aim at giving an overview of the chromatographic and electrophoretic approaches including recent examples of their use.

General considerations in sample preparation

Nanodiscs are assembled by mixing MSPs with detergent solubilized phospholipids after which the self-assembly process is initiated by detergent removal. For the reconstitution of nanodiscs containing membrane proteins, the target membrane protein is simply included in the reconstitution mixture, usually in a purified and detergent solubilized state. A range of different detergents has been applied in the reconstitution of nanodiscs, including mixed detergents in the cases where the primary detergent

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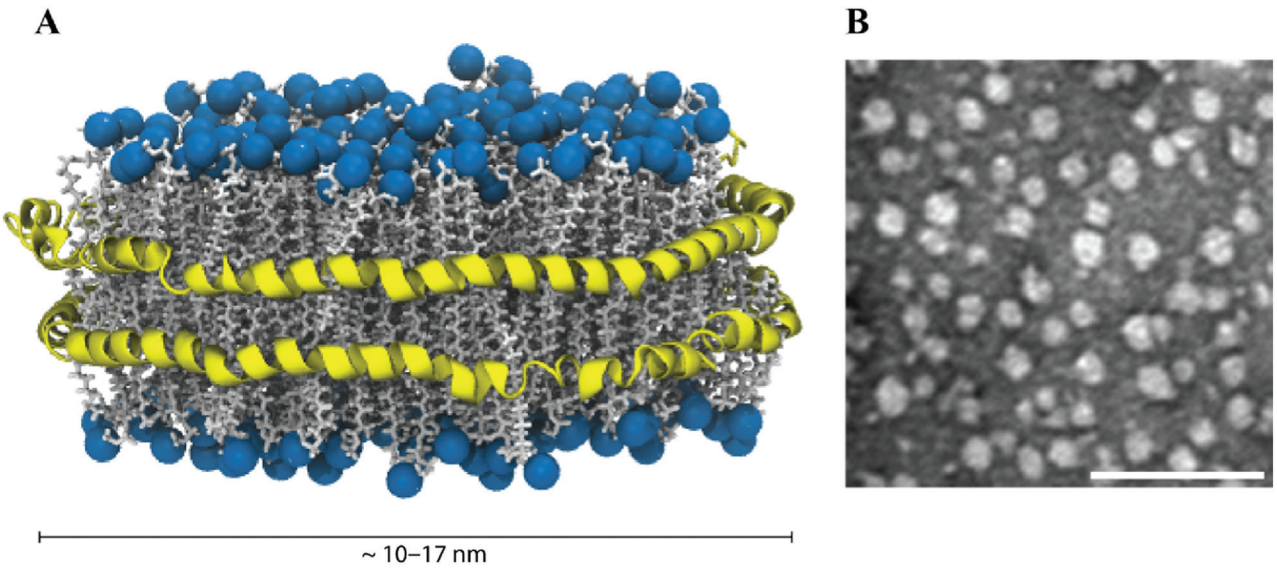

Figure 1 Phospholipid nanodiscs.
(A) Illustration of a nanodisc with two copies of MSP (yellow) MSP1D1 surrounding the hydrocarbon side chains (white) of a lipid bilayer of POPC (lipid head groups are modeled as blue spheres). The model was prepared using Visual Molecular Dynamics (VMD) (Humphrey et al. 1996). The coordinates for MSP1D1 were adopted and modified slightly from a cryo-electron microscopy (EM) solved structure of the bacterial ribosome-SecYEG complex in a nanodisc by Frauenfeld et al. (2011). The POPC bilayer was created using the membrane builder tool in VMD. (B) Negatively stained EM images of nanodiscs prepared using the MSP1E3 scaffolding protein. For experimental details, see Justesen et al. (2013a). Scale bar represents 100 nm.

for lipid solubilization is harmful to the target membrane protein (Supplementary Table 1). A critical step in nanodisc reconstitution is the complete solubilization of the lipid/detergent mixture into mixed micelles before the addition of MSPs (Bayburt and Sligar 2010). The removal of detergents to trigger self-assembly is often achieved using either dialysis or absorption to porous polystyrene beads, such as Biobeads SM-2 or Amberlite XAD2. A recent work has also shown the use of cyclodextrins for this aim (Pandit et al. 2011). In some cases, hydrophobic polystyrene beads have been shown to be less applicable since they may also absorb MSPs, the target protein or lipids (Roos et al. 2012). In these cases, the alternative option of dialysis might prove a better choice, although the feasibility of this option depends on the critical micelle concentration (CMC) of the used detergent; low CMC detergents cannot be easily removed by dialysis. Furthermore, the detergent removal rate can affect the overall yield of homogenous nanodiscs (Denisov et al. 2004).

Of particular importance for the assembly of proper nanodiscs and successful reconstitution of membrane proteins is the optimal lipid/MSP/membrane protein stoichiometry (Mörs et al. 2013). The reconstitutions using ratios outside the optimal range result in aggregates of lipids or MSP as well as the possibility of retarded disc shapes or other lipoprotein particles. The overall size of nanodiscs can be controlled by varying the length of the MSPs with a range of different constructs developed to suit the reconstitution of larger membrane proteins or to allow for multiple copies in single nanodiscs. MSPs containing inserts of one, two, and three additional 22-mer amphipathic helical repeats in the central position of the protein have been created by Denisov et al. (2004) in addition to MSPs comprising two copies of the original MSP created by Grinkova et al. (2010). Using these constructs, a nanodisc with diameters ranging from 9.8 to 17 nm with up to about 300 lipids per leaflet can be assembled. Furthermore, truncated constructs lacking combinations of the helical repeats 2 and 4–6 out of the 10 helical repeats in MSP1D1 have been generated, which facilitate the analysis of nanodisc-embedded membrane proteins by NMR spectroscopy (Hagn et al. 2013). The optimal lipid/MSP ratios for the reconstitution of empty nanodiscs have been determined experimentally for different MSP and lipid species (Bayburt et al. 2002, Denisov et al. 2004). Through established reconstitution protocols (e.g., see Sligar Lab homepage; http://sligarlab.life.uiuc.edu/nanodisc/protocols.html) (Ritchie et al. 2009a, Dalal and Duong 2010), the assembly of monodisperse nanodisc preparations with size distributions as low as 3% (Denisov et al. 2004) can be performed reproducibly. Often, the reconstitutions in the presence of membrane proteins are performed

under conditions that result in a large excess of empty nanodiscs. In these cases, the optimal lipid/MSP ratio is usually identical to a similar reconstitution of nanodiscs without the membrane protein. This approach facilitates the reconstitution of membrane proteins as monomers but creates the need for subsequent purification steps to separate empty from membrane protein-containing nanodiscs. In the following, we will discuss several chromatographic and electrophoretic techniques to achieve this separation, which often returns valuable information on the nanodisc characteristics such as size and charge.

Size exclusion and affinity chromatography

The ability to obtain samples with a narrow size distribution is an essential characteristic of nanodiscs. For several biophysical methods (e.g., small-angle scattering techniques), this monodispersity is key and often a requirement for a successful analysis. Consequently, the methods for determining the dimensions and size distribution of nanodiscs are important tools for the nanodisc sample preparation. Often, the purification and analysis of nanodiscs based on their dimensions also allows for the separation of empty from membrane protein-containing nanodiscs as well as the verification of proper reconstitution based on the expected dimensions.

The generic approach for the size-dependent nanodisc purification after reconstitution is size exclusion chromatography (SEC). This relatively simple technique has been used from the very beginning of nanodisc reconstitution and remains an almost mandatory step in the nanodisc sample preparation. SEC is well suited to remove any aggregates from nanodisc reconstitution and for the estimation of the dimensions and homogeneity of the samples. The large majority of published work on nanodiscs involving SEC applies a Superdex 200 column (Supplementary Table 1). With a separation range of 10,000–600,000 kDa, this column is well suited for purifying and analyzing nanodiscs, with empty discs having molecular weights in the range of 142 kDa (MSP1), 175 kDa (MSP1E1), 217 kDa (MSP1E2), and 254 kDa (MSP1E3) [calculated for nanodiscs assembled from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)] (Denisov et al. 2004, Ritchie et al. 2009b). By running a range of standard proteins of known Stokes radius, it is possible to calibrate the SEC column and to estimate the apparent Stokes radius of the nanodiscs (Bayburt et al. 2002, Beales et al. 2013, Justesen et al. 2013a). Furthermore, dynamic light scattering, which is also available in combination with SEC (termed absolute SEC) as well as analytical ultracentrifugation, has been used for measuring the dimension and molecular weight of nanodiscs (reviewed in Inagaki et al. 2013).

SEC is also a useful tool for the validation of correct nanodisc assembly and membrane protein reconstitution. Typically, a reinjection of the fractions covering the nanodisc peak is performed to confirm the stability of the reconstituted samples (Bayburt and Sligar 2003). The homogeneity of the nanodiscs is verified from the presence of a single elution peak, and membrane protein reconstitution is simply validated from the coelution of the MSPs and the target membrane protein. This is often analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (see below), although an alternative approach is the use of fluorescent-labeled membrane proteins, allowing for the absorption to be monitored directly in the chromatogram. A similar approach can also be applied to verify a proper nanodisc assembly, using fluorescent-labeled phospholipids, thereby monitoring the coelution of lipids and MSPs (e.g., see Supplementary Figure S-2 in Justesen et al. 2013b).

Unfortunately, SEC does often not provide a sufficient high resolution for the separation of empty from membrane protein-containing nanodiscs, particularly in the cases of membrane proteins with minor extramembrane domains. This can, for example, been seen in various reports on the nanodisc-embedded bacteriorhodopsin (Bayburt and Sligar 2003, Bayburt et al. 2006, Shenkarev et al. 2013), a ~26 kDa seven-transmembrane integral membrane protein with only small extramembrane loops, resulting in only a small difference in the Stokes radius between empty and membrane protein-containing nanodiscs. Contrary to this example are the reconstitutions of the ~60 kDa *Escherichia coli* Tar chemoreceptor (Boldog et al. 2006) and the ~181 kDa human platelet glycoprotein Ib-IX complex (Yan et al. 2011), both comprising large extramembrane regions resulting in a clear separation when analyzing using SEC.

Despite it being difficult to set a clear boundary for the useful application of SEC in the separation of empty and membrane protein-containing nanodiscs, a general prerequisite is a substantial difference being present between their respective hydrodynamic radii. In the cases where this does not apply, the methods independent of the size difference between empty and membrane protein-containing nanodiscs are required, which is typically achieved utilizing affinity chromatography (Baas et al. 2004). A prerequisite for using this approach is the addition of an affinity tag to the target membrane protein, for

example, polyhistidine or the biotinylation for Ni^{2+}-affinity or biotin-avidin affinity purification, respectively. In the cases of the Ni^{2+}-affinity-mediated separation of empty and membrane protein-containing nanodiscs, several MSP constructs have been generated containing either a tobacco etch virus (TEV) or a Factor X recognition site, thus allowing for the removal of the polyhistidine tag on the MSPs by treatment with TEV protease or Factor Xa, respectively (Grinkova et al. 2010). The membrane protein-containing nanodiscs are separated from empty nanodiscs through the binding of the target membrane protein to the affinity matrix after nanodisc reconstitution. This results in a flow-through containing empty nanodiscs and allows for the elution of membrane protein-containing nanodiscs. Since no information on the nanodisc assembly is obtained during affinity purification, this approach is usually used in conjugation with SEC analysis to verify a proper nanodisc assembly, either prior to or after the separation of empty from membrane protein-containing nanodiscs.

In addition to the separation of empty and membrane protein-containing nanodiscs, affinity purification is also used for the co-reconstitution of more than one membrane protein into a single nanodisc. This can be achieved by the addition of different tags on each target membrane protein and nanodisc reconstitution followed by the consecutive affinity purifications for each membrane protein. One example using this approach is the co-reconstitution of a histidine-tagged cytochrome P450 CYP3A4 with a cytochrome P450 oxidoreductase (POR) into nanodiscs assembled with the scaffold protein MSP1E3D1 by Denisov et al. (2007). The authors first removed the polyhistidine tag on the MSP1E3D1 prior to reconstitution, thereby allowing the use of Ni^{2+}-affinity chromatography to remove the empty and only POR-containing nanodiscs. The remaining mixture of nanodiscs containing either only CYP3A4 or both CYP3A4 and POR was subsequently applied to an ADP affinity column, binding the POR enzyme and allowing for a final elution of the nanodisc assemblies containing both CYP3A4 and POR.

A final example for the use of affinity purification in the nanodisc preparation is its combination with nanodisc reconstitution in what is termed on-column reconstitution (Katayama et al. 2010, Puthenveetil and Vinogradova 2013). Here, either the MSP or the target membrane protein is immobilized on an affinity resin, after which the remaining of the reconstitution components such as lipids and detergents are added. Following normal reconstitution protocols, the affinity resin is washed, and depending on the immobilized component, either a mixture of empty and membrane protein-containing nanodiscs or only membrane protein-containing nanodisc can be eluted. This approach allows for a single-step nanodisc reconstitution and purification. Despite the uncertainties in matching optimal reconstitution conditions such as MSP concentration on the column, recent reports have shown it to be feasible for the preparation of monodisperse nanodisc samples (Puthenveetil and Vinogradova 2013).

Free flow electrophoresis

Free flow electrophoresis (FFE) is a technique designed to separate the analytes based on their electrophoretic mobility, which is applicable on both an analytical scale and a preparative scale. The separation is performed in aqueous media in the absence of a matrix, thereby reducing the loss of the material due to unspecific binding or denaturing effects, which in turn results in the possibility to obtain high yields (>80%) and preserve the sample activity (Justesen et al. 2013b). Consequently, FFE can be used both as a standalone purification of assembled nanodiscs or in conjunction with downstream applications or analysis. Usually, a 100 µl sample (0.5–5 mg protein/ml) is loaded for a single analytical separation, although this volume depends on the separation and injection flow rates and therefore may vary depending on the experimental setup. During separation, the sample is streamed into a planar flow chamber. An electric field is applied perpendicularly to the direction of flow, deflecting analytes according to their electrophoretic mobility, as they travel through the flow channel. The traditional use of FFE involves the application of a continuous buffer flow and electrical field. However, this approach suffers from a number of effects that result in band broadening and thus a lower resolution of the FFE separation. Such effects includes diffusional broadening, hydrodynamic broadening, electromigration dispersion, and heat broadening (Shao et al. 2012). A number of developments have been made in order to counter these effects, including the use of concentrating modes such as isoelectric focusing or isotachophoresis, operated with pH gradients or steps in the separation buffer, respectively (Hoffstetter-Kuhn et al. 1990, Moritz and Simpson 2005). For the separation of nanodisc samples, a noncontinuous mode of FFE, termed free flow interval zone electrophoresis (IZE), was recently applied (Justesen et al. 2013b). In this mode, the electric field is applied for a short period of time during the run, while the sample application and the final elution of the separated analytes are performed with the electrical field

switched off. A typical separation using this mode takes around 10–15 min and can immediately be repeated batch-wise until the desired amount of the separated sample is reached. Compared to the continuous mode, IZE results in an increased resolution due to the elimination of the hydrodynamic broadening effect. Furthermore, the IZE can be performed in relative simple buffer systems at a variety of pH values. The approach is applicable for both highly and close to neutral charged membrane proteins with distinctly different structures and properties. Accordingly, IZE is also applicable following the modification of the MSP or membrane proteins, for example, by the addition of affinity tags to align the discs on the surfaces or by the addition of fluorescent tags to monitor the conformational changes. Figure 2 shows an example of free flow IZE performed at a pH step gradient ranging from pH 4–9, allowing for the high-resolution separations of the mixtures of nanodiscs assembled from two different MSPs. The possibility to independently vary several parameters for the nanodisc self-assembly process, such as lipid charges and disc sizes, opens up a range of different strategies to be employed in the free flow IZE-based separation. The use of step gradients in free flow IZE mode (IZE-pH) has the benefit that the modified pH values of the separation buffers increase the effect of band sharpening at each pH step the analyte passes. This effect happens as the analytes are slowing down in their electromigrational movement on the pH steps, causing the remaining protein peak to catch up and thus sharpening the bands.

Taken together, the main advantages of FFE-based nanodisc purification compared to the commonly applied techniques such as SEC and affinity chromatography is the absence of a matrix and short separation time, resulting in a quick and gentle treatment of the nanodisc samples. This in turn minimizes the risk of denaturing the samples. Since FFE is based on the migration of charged or chargeable analytes in an electrical field, a successful separation is based on the charge difference rather than on the hydrodynamic radius as for SEC, which is beneficial in the cases of separation of similar-sized nanodisc complexes. Naturally, this also limits the FFE application to samples with sufficient differences in charges.

Gel electrophoresis

Gel electrophoresis is extensively used to verify the nanodisc formation and successful coinsertion of the membrane protein. Denaturing SDS-PAGE uses polyacrylamide as a sieving matrix to separate the SDS-bound proteins by their molecular weight. To estimate the number of reconstituted proteins per disc, gel densitometry by quantitative Coomassie staining can be used (Bayburt et al. 2006, Shaw et al. 2007). Furthermore, chemical crosslinking has been employed to analyze the oligomerization status of nanodisc-reconstituted membrane proteins. Chemical crosslinking reagents create covalent links between the adjacent proteins that will therefore migrate as one single complex during SDS-PAGE. The number of protomers is deduced from the molecular mass of each monomer compared to the molecular mass of the crosslinked complex. In the case of nanodiscs, however, chemical crosslinking results additionally in the formation of dimeric forms of MSP and membrane protein/MSP assemblies that may complicate the interpretation of the results. It is therefore helpful to perform an additional analysis using Western blotting with specific antibodies against the MSP and membrane protein, respectively (Lyukmanova et al. 2012, Justesen et al. 2013a).

Native PAGE, another gel electrophoresis method, is used to study the intact nanodiscs under nondenaturing conditions, thereby providing a detailed information about their homogeneity. In a similar manner to SDS-PAGE, nanodiscs are separated by size, but the milder Coomassie dye (blue native PAGE) or a noncolored anionic reagent (clear native PAGE) is used instead of SDS to provide the necessary negative charge for the electrophoretic separation under native conditions. A special advantage of clear native electrophoresis is the possibility for in-gel catalytic activity assays and in-gel detection of fluorescent-labeled proteins. Yet care must be taken when the molecular masses of the nanodiscs are estimated from their migration relative to the standard native marker proteins. The migration of the nanodiscs is strongly influenced by the lipids and membrane protein (Crichton et al. 2013, Justesen et al. 2013a).

Summary

The heterogeneity of nanodisc preparations is a well-known phenomenon and it is often a challenge to identify and purify the definite subpopulations. Each analytical method presented here has particular advantages but also limitations. In general, SEC and affinity chromatography are still the most widely used techniques for the analysis and separation of nanodiscs. Both techniques provide efficient tools for the purification and characterization of nanodiscs, especially when assembled with trace

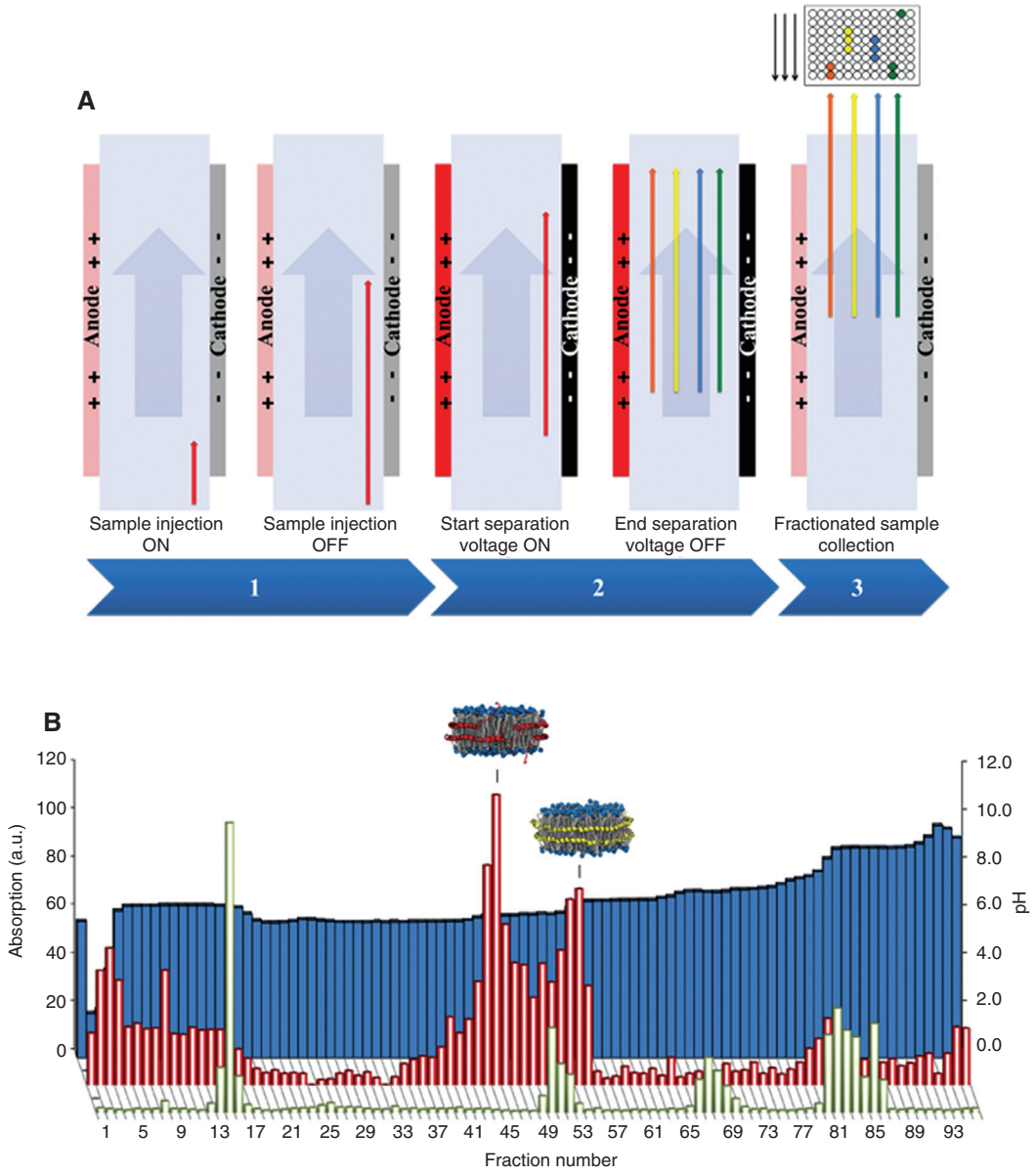

Figure 2 Free flow IZE for the separation of nanodiscs.
(A) Schematics of the free flow IZE separation process. (Step 1) The sample is injected into the continuous laminar flow and allowed to continue into the chamber. (Step 2) The electrical field is applied in an optional time interval depending on the electric mobility of the compound of interest. (Step 3) After the separation of the sample, the fractions are collected into a 96-well plate and are available for analysis. (B) Nanodisc separation in pH step gradients using free flow IZE. The nanodiscs were assembled from neutrally charged phosphatidylcholine and two sets of MSP1D1 (one with and one without a histidine tag) as described previously (Justesen et al. 2013b) and separated in the pH range of pH 4.0–9.4. The separation is based on a greater electrophoretic mobility resulting from the negative charge of histidine tag MSP1D1. The mobility of the individual nanodiscs (red) toward the anode was monitored by the absorption at 280 nm. On the top of the nanodisc samples is an illustration of a nanodisc composed of MSP with (red MSP) and without (yellow MSP) a histidine tag. The blue line indicates the pH profile of the FFE run. A mixture of six pI markers (yellow) with differing electric mobility was included, demonstrating the boundaries toward the cathodic and anodic stabilization buffers.

amounts of fluorescence lipids and fluorescent-tagged membrane proteins.

As shown here and by another study (Justesen et al. 2013b), FFE can greatly improve the separation of nanodisc preparations, thus laying the foundation for a much more efficient identification, purification to homogeneity, and detailed characterization since this technique is compatible with the most widely used downstream analysis methods such as gel electrophoresis and chromatography-based techniques.

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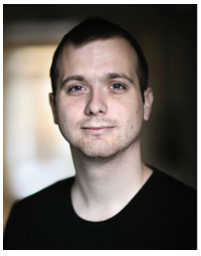

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