Fluidic Sciences

APPLICATION NOTE

Purification-free affinity and concentration measurement of membrane-protein targets

One of the biggest challenges to studying membrane proteins is their quantitative characterization under native-like conditions that preserve their structural and functional integrity. Here, we introduce an integrated preparative and analytical method that enables researchers to simultaneously measure both the concentration of an endogenous membrane-protein target and its binding affinity to a specific ligand in a native lipid-bilayer environment directly extracted from crude cellular membranes. To demonstrate our approach, we first used the nanodisc-forming polymer glyco-DIBMA to create a native membrane-protein library of a breast cancer cell line. Using Microfluidic Diffusional Sizing (MDS) on the Fluidity One-M, we then determined both the concentration of the endogenous oncoprotein HER2 and its affinity to trastuzumab, a therapeutic HER2-specific antibody. The combination of native membrane-protein libraries with MDS provides quantitative information on membrane proteins without the need for laborious and costly protein-purification campaigns. The method is straightforward to adopt in standard research laboratories, takes only a few hours to complete, and has the potential to be expanded from cell lines to tissues and tumor biopsies.

Introduction

Membrane proteins are involved in a wide variety of cellular processes such as transport, signaling, immune recognition, and adhesion, and thus provide immense opportunities for new therapeutic approaches. This is further evidenced by the over-representation of membrane proteins as targets of existing therapeutics, with >60% of drugs targeting membrane proteins despite them making up ~25% of the proteome. Despite their potential, research on membrane proteins is still seen as an expert field which requires significant specialist expertise, time, and money.

Our newly developed Membrane-protein Affinity and Concentration (MAffCon) Assay bridges this gap by providing a straightforward method for quantitative analysis of membrane proteins (Figure 1). The MAffCon Assay solubilizes membrane proteins directly from cells which can then be analyzed by Microfluidic Diffusional Sizing (MDS) using the Fluidity One-M—without having to screen detergents or carry out any purification. The measurement on the Fluidity One-M then provides the binding affinity of, for example, an antibody and the concentration of the target membrane protein.

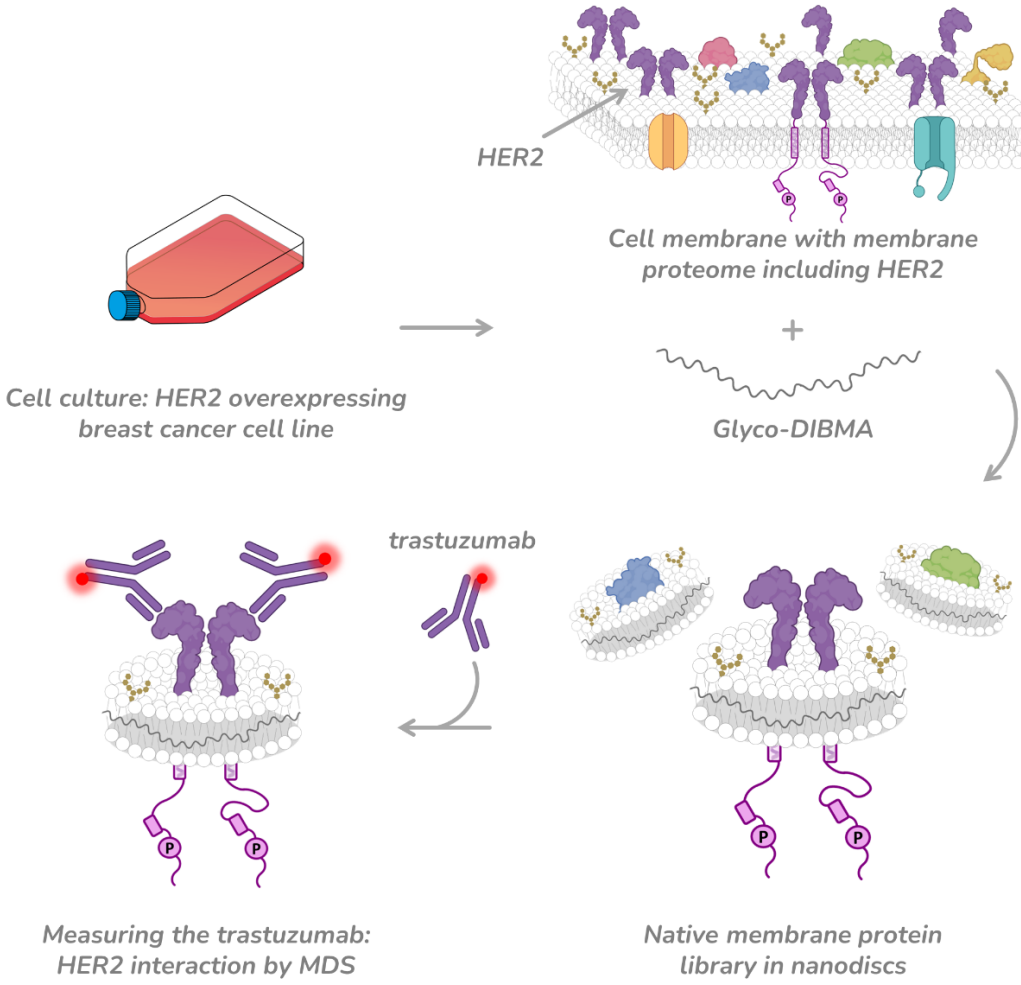

Figure 1. Overview of the purification-free workflow offered by the MAffCon Assay.

Here, we use the MAffCon Assay to study binding of fluorescently labeled trastuzumab to HER2 extracted from breast cancer cells. HER2 is a single-pass transmembrane protein whose overexpression in breast cancer is associated with poor clinical outcomes, while trastuzumab is a therapeutic antibody drug targeting HER2. Our data is consistent with literature values of trastuzumab affinity and the affinity to an extracellular HER2 domain used as a control. Moreover, the HER2 concentration determined by the MAffCon Assay provides a straightforward route to quantify HER2 expression.

Crucially, the assay only takes 2-3 hours, is purification-free, provides universally comparable K_D values, determines the concentration of active target, and stabilizes targets in a lipid bilayer.

Results

The MAffCon Assay is split into two main components: 1) the preparation of native membrane-protein libraries, and 2) the analysis performed on the Fluidity One-M. For sample preparation, we generated a native membrane-protein library by adding the amphiphilic copolymer glycol-DIBMA to the HER2-overexpressing breast cancer cell line HCC1419 (Figure 2).

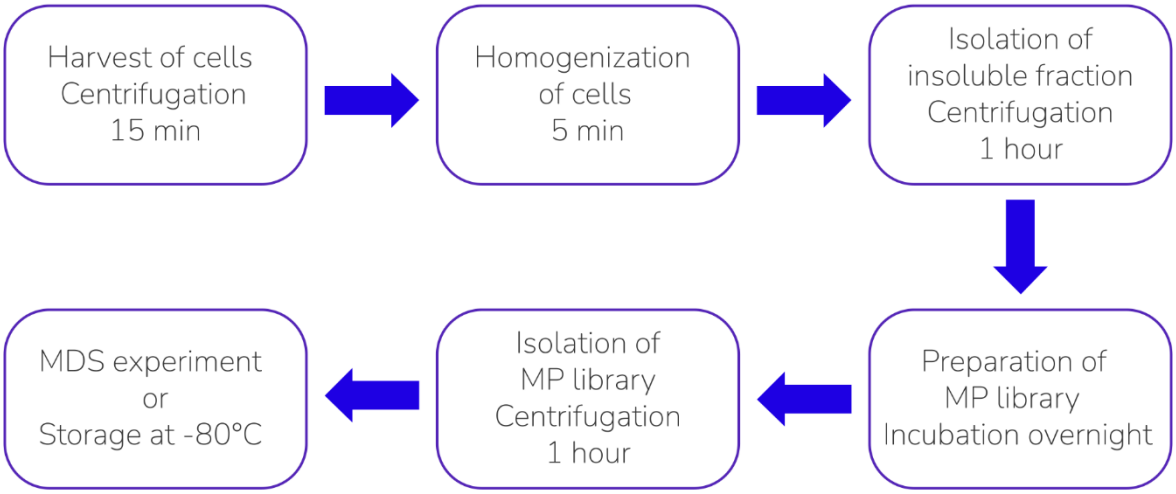

Figure 2. Workflow and timings to prepare native membrane-protein library using Glyco-DIBMA

For analysis on the Fluidity One-M, fluorescently labeled trastuzumab at two different concentrations was mixed with eight different dilutions of the native membrane-protein library obtained from the HER2-overexpressing HCC1419 cells (Figure 2). Trastuzumab binding was detected based on the increase in size, and both binding curves were analyzed globally to determine K_D and target protein concentration. As a control, we also determined the affinity of trastuzumab to the extracellular domain of HER2 which yielded the same value as that of the HCC1419 membrane-protein library.

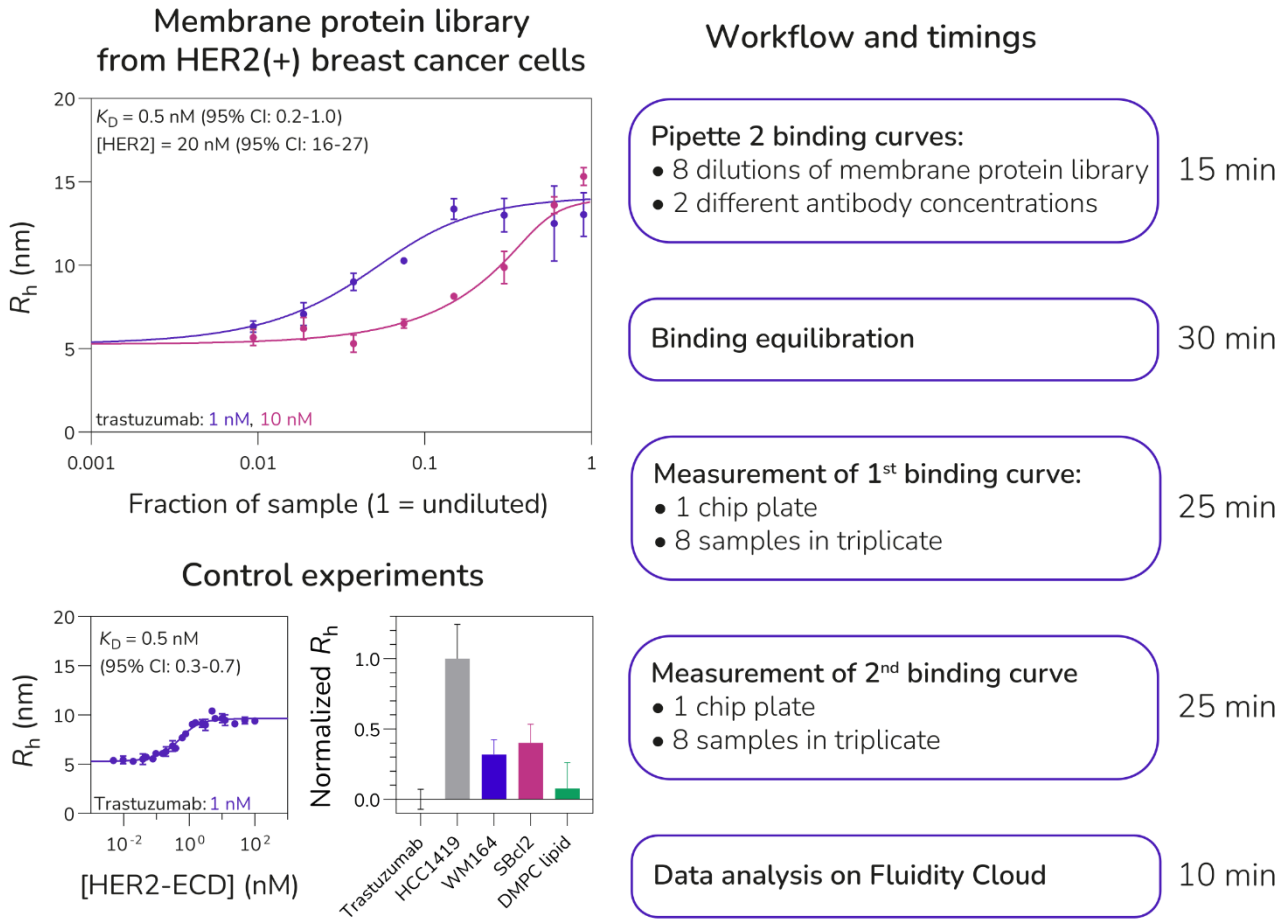

Figure 3. Workflow and timings to prepare native membrane-protein library using Glyco-DIBMA.

As negative controls, we tested trastuzumab binding to native membrane-protein libraries obtained from two melanoma cell lines (WM164 and SBcl-2) which express HER2 at basal levels. Both melanoma cell lines resulted in only 30%- 40% of the size change measured for HCC1419 cells (all three samples had the same total protein concentration) demonstrating the specificity of the assay. To additionally check for any non-specific antibody binding to glyco-DIBMA, we challenged fluorescently labeled trastuzumab with glyco-DIBMA nanodiscs composed of the synthetic lipid DMPC which showed very low levels of binding.

Conclusion

Here, we present the Membrane-protein Affinity and Concentration (MAffCon) Assay which provides purification-free, quantitative analysis of membrane proteins in a native lipid bilayer environment. The MAffCon Assay yields the absolute target concentration within a native membrane-protein library directly extracted from cells or tissues and the ligand binding affinity to that target within a single experiment. The assay delivers next-day results, works with as few as 3 x 10^6 cells, applies to endogenous targets as well as recombinantly expressed proteins, and requires no expert experience in membrane-protein research.

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Methods

Fluorescent labeling of trastuzumab

Trastuzumab was fluorescently labeled with Alexa Fluor™ 647 NHS ester (Thermo Fisher). A solution containing 100 µg of antibody was mixed with dye at a three-fold molar excess in the presence of $NaHCO_3$ (Merck) buffer at pH 8.3 and incubated at 4 °C overnight. Unbound label was removed using a Zeba™ desalting chromatography cartridge (7K MWCO, 1 mL, Thermo Fisher). Labeled and purified trastuzumab was stored at −80 °C in PBS pH 7.4 containing 10% (w/v) glycerol as a cryoprotectant.

Preparation of polymer stock

Glyco-DIBMA was dissolved in sterile filtered Tris buffer (0.8 g polymer in 4 mL 150 mM NaCl, 50 mM Tris, pH 7.4) and dialyzed overnight (MWCO 3 kDa). The mass concentration of the polymer was determined using UV-VIS absorbance at 220 nm (ε = 1.62 L/g.cm). Dissolved polymer stock can be stored at 4 °C for at least 6 months.

Preparation of native membrane-protein libraries

Cells were grown to 90–95% confluency in a T175 cell culture flask (yield ~1 x 10^7 cells). Cells were washed twice with DPBS (GIBCO) and once with sterile filtered Tris buffer (150 mM NaCl, 50 mM Tris, pH 7.4). Then, 5 mL of sterile filtered Tris buffer containing 1 x cOmplete protease inhibitor was added to the cell culture flask and cells were harvested using a sterile cell scraper. Cells were collected and homogenized with a Potter-Elvehjem tissue grinder on ice. The cell homogenate was split into aliquots corresponding to 3 x 10^6 cells and centrifuged at 200,000 g for 30 min.

For each aliquot, the pellet was weighed and resuspended in sterile filtered Tris buffer containing 1 x cOmplete at a final wet biomass concentration of 60 mg/mL. 0.05% benzonase was added followed by 15 min incubation. Glyco-DIBMA stock was added to reach a mass ratio of 1.0 and the resulting mixture was incubated overnight at 4 °C under shaking.

After overnight incubation, the sample was centrifuged at 200,000 g for 30 min followed by careful removal of the supernatant containing the entire membrane proteome in nanodiscs. The total protein concentration was determined by bicinchoninic acid (BCA) assay. The sample was then aliquoted into volumes of 50 µL, snap frozen in liquid nitrogen and stored at -80 °C until further use.

Binding of trastuzumab to native membrane-protein libraries

Fluorescently labeled antibody at concentrations of either 1 nM or 10 nM was mixed with various dilutions of native membrane-protein libraries (90%, 60%, 30%, 15%, 7.5%, 3.75%, 1.88% and 0.94%) and incubated on ice for at least 30 min. Binding was assessed by measuring the hydrodynamic radius (R_h) by Microfluidic Diffusional Sizing on the Fluidity One-M, by loading 4 µL of reaction mixture in the sample ports of the microfluidic chip device. Native membrane-protein libraries at the same concentration but without labeled antibody were loaded in the auxiliary port.

The binding affinity, K_D, and the active concentration of target were determined from the primary data using the cloud-based analytical suite.

Negative controls

Control experiments were performed using native membrane-protein libraries obtained from melanoma cell lines WM164 and SBcl-2 as well as glyco-DIBMA nanodiscs formed from the synthetic lipid DMPC.

Acknowledgements

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Equations

$$f_d = \rho_f + \frac{[B]}{[L]}(\rho_b - \rho_f)$$

F_d	fraction of labeled species that diffused into the auxiliary flow of the microfluidic chip
[L]	total concentration of labeled species
[B]	equilibrium concentration of bound labeled species
ρ_f	fraction of free labeled species
ρ_b	fraction of bound labeled species

$$[B] = \frac{[L] + \alpha[U] + K_D - \sqrt{([L] + \alpha[U] + K_D)^2 - 4\alpha[L][U]}}{2}$$

[U]	total concentration of binding sites in the unlabeled species
α	fractional concentration of unlabeled species in the binding measurement
K_D	dissociation constant (affinity)

About us

It's not just the proteins that make life, it's the interactions among them. Here at Fluidic Sciences, we make protein interaction analysis easy and robust by developing transformative in-solution technologies and accessible instruments that help scientists quickly and accurately understand how proteins truly interact.

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