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Detergent screening of a GPCR using serial and array biosensor technologies

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Abstract

We describe the benefits and limitations of two biosensor approaches for screening solubilization conditions for G-protein-coupled receptors. Assays designed for a serially processing instrument (Biacore 2000/3000/T100) and an array platform (Biacore Flexchip) were used to examine how effectively 96 different detergents solubilized the chemokine receptor CCR5 while maintaining its binding activity for a conformationally sensitive Fab (2D7). Using the serial-processing instrument, we were able to analyze three samples in each thirty-minute binding cycle, thereby requiring ~24 hours to screen an entire 96-well plate of conditions. But, in-line capturing allowed us to normalize the 2D7 binding responses for different receptor capture levels. In contrast, with the array system we could characterize the effects of all 96 detergents simultaneously, completing the assay in <1 hour. But the current array technology requires that we capture the GPCR preparations off line, making it more challenging to normalize for receptor capture levels. Also, the array platform is less sensitive than the serial platforms, which limits the size of the analyte to larger molecules (>5000 Da). Overall, the two approaches proved highly complementary: both assays identified identical detergents that produced active solubilized CCR5, as well as those detergents that either were ineffective solubilizers or inactivated the receptor.

Keywords

array; Biacore; CCR5; Flexchip; GPCR; optical biosensor; surface plasmon resonance

Introduction

Surface plasmon resonance (SPR) biosensors can be used to identify suitable conditions for isolating G-protein-coupled receptors (GPCRs) from membranes while preserving the receptors' abilities to bind conformationally sensitive ligands. For example, we have used SPR in the past to screen GPCR solubilization, purification, and crystallization conditions [1–3]. We identified components of solubilization and crystallization buffers that increased a GPCR's stability and we optimized affinity-purification methods to yield highly active receptor.

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Here we outline two biosensor-based assays that reveal how different detergents affect the yield and activity of solubilized GPCRs. We used CCR5 as the model system since we have access to high-quality reagents and binding partners. Our CCR5 construct is C-terminally tagged with a peptide sequence recognized by 1D4 antibody [4], which stably captures the receptor yet can be regenerated at the end of each binding cycle. To probe the conformation of CCR5, we used a Fab of the 2D7 antibody.

The detergent screening methods were developed using Biacore 2000 and Flexchip platforms. The microfluidics system in the Biacore 2000 can test one analyte across a reference and up to three reaction surfaces [5]. After each binding cycle the surfaces are regenerated and new aliquots of receptor are captured. While serial sample processing decreases throughput, this instrument's sensitivity (it is routinely used to examine molecules <500 Da), combined with its ability to monitor both ligand capture and analyte binding, makes it useful for receptor screening. In the Biacore Flexchip array platform, an analyte is flowed across a matrix of ligand "spots" within a single large-format flow cell which can significantly improve ligand throughput [5,7]. But array biosensors have lower sensitivity than traditional serial platforms; Flexchip's limit of detection is approximately 5000 Da. And, at present ligands are spotted off line so capture levels, for example, cannot be directly quantitated.

Our screening methods took advantage of the unique features of these two platforms. With Biacore 2000 we established the correlation between ligand capture and Fab binding levels. For Biacore Flexchip we devised a rapid assay that should prove particularly useful for GPCRs that are less stable than our CCR5 construct. Using either approach, we could bin the detergents into three groups: those that did not solubilize CCR5, those that solubilized but inactivated the receptor, and those that yielded active solubilized receptor.

Materials and Methods

Materials

The Biacore 2000 and Flexchip optical biosensors, as well as instrument-specific consumables (e.g., sensor chips, affinity chip kits, amine-coupling kits, and standard 96-well plates and covers), were obtained from GE Healthcare/Biacore (Uppsala, Sweden). Cf2Th canine thymocyte cells overexpressing the human chemokine receptor CCR5 tagged with a C-terminal linear peptide tag (C9: TETSQVAPA) were propagated by the National Cell Culture Center. Purified mouse anti-human CD195 (CCR5) monoclonal antibody 2D7 was provided by the National Institutes of Health AIDS Research and Reference Reagent Program and purchased from BD Biosciences (San Jose, CA, USA); the C9 tag-recognizing antibody 1D4 was purchased from the University of British Columbia (Technology Transfer Office, University of British Columbia, Vancouver, British Columbia, Canada). Detergents were purchased from Anatrace (Maumee, OH, USA), DOPC:DOPS (7:3) lipid mixture from Avanti Polar Lipids (Alabaster, AL, USA), polyethylene glycol (PEG) 8000 from Promega Corp. (Milwaukee, WI, USA), ImmunoPure® Fab Preparation Kit from Pierce (Rockford, IL, USA), and protease inhibitor tablets from Roche Diagnostics (Indianapolis, IN, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Rockford, IL, USA).

Preparation of 2D7 Fab

One mL of clone 2D7 mAb (BD Pharmingen cat # 555991, 0.5 mg/mL in 50 mM tris 150 mM NaCl, pH 8.5, 0.09% sodium azide; conformational sensitivity demonstrated by Navratilova et al. [1]) was dialyzed extensively against 20 mM sodium phosphate, 10 mM EDTA, pH 7.0 at 4°C prior to Fab preparation. 2D7 Fab fragments were obtained and purified initially using the ImmunoPure® Fab Preparation Kit (Pierce cat #44885). Using the Biacore 2000, fractions

collected from the Protein A column were tested for binding to 1D4-captured CCR5 solubilized as described in reference 8. Fractions that bound CCR5 were passed over a sizing column to ensure purity of the 2D7 Fab. The activity of these post-sizing column fractions were confirmed by binding to the CCR5 surface. Fractions containing 2D7 Fab were stored at 4°C until use.

Immobilization of anti-C9 1D4 mAb on CM5 and Flexchip surfaces

Using HEPES-buffered saline (HBS: 10 mM HEPES, 150 mM NaCl, pH 7.4) as the running buffer, 1D4 antibody (diluted in 10 mM sodium acetate pH 5.5) was immobilized at 25°C via standard amine-coupling chemistry to densities of approximately 7000 RU on the four flow cell surfaces of a CM4 sensor chip. Using the same chemistry, 1D4 was immobilized across a Flexchip prototype dextran-coated affinity chip.

Preparation of detergent and lipid/cholesterol solutions

Stock solutions of detergents were prepared at 5% in water and stored at 4°C until use. For solubilization trials, 20 μ L of each detergent stock solution was dispensed into individual wells of a 96-well plate.

Dry films of DOPC/DOPS lipid were prepared as described by Navratilova *et al.* [8]. Immediately prior to receptor solubilization, one mL of solubilization buffer base (20 mM tris, 0.1 M $(\text{NH}_4)_2\text{SO}_4$, 10% glycerol, 10% PEG 8000, 0.07% cholesteryl hemisuccinate tris salt (CHS), pH 7.0) was added to one aliquot of film, after which the solution underwent four vortex-freeze-thaw cycles and was then diluted with chilled solubilization buffer (20 mM tris, 0.1 M $(\text{NH}_4)_2\text{SO}_4$, 10% glycerol, 10% PEG 8000, 0.07% CHS, pH 7.0 with one protease inhibitor tablet per 50mL) to yield a lipid concentration of 0.33 mM.

Solubilization of C9-tagged CCR5

All steps were performed on ice or in a cold room. Approximately 6×10^6 CCR5-expressing cells were resuspended in three mL of the lipid-containing solubilization buffer, sonicated using a Soniprep 150 probe sonicator (six 1-s pulses), and diluted to 20 mL with lipid-containing solubilization buffer. 180 μ L of this cell suspension was added to each well of a 96-well plate that contained 20 μ L detergent (see above). After sealing, the plate was gently spun on a vertical rotator for six hours and then centrifuged. Supernatants were transferred to a new 96-well plate. As controls, each detergent screen included positive (buffer containing 0.33% CHAPS/0.33% DDM) and negative (no detergent added) controls.

CCR5 capture and 2D7 Fab binding using Biacore 2000

Receptor capture and Fab binding studies were performed at 25°C using 50 mM HEPES, 150 mM NaCl, 0.02% CHS, 0.1% DDM, 0.1% CHAPS, 50 nM DOPC/DOPS (7:3), 0.2 mg/mL BSA, pH 7.0 as the analysis buffer. In one binding cycle, three CCR5 preparations were captured on individual 1D4-coated flow cell surface, after which 2D7 Fab (100 nM) was injected for four minutes across the reference (1D4 alone) and three CCR5 surfaces. After a wash phase of one minute, the surfaces were regenerated with 1% OGPS/10 mM NaOH. The standard CCR5 solubilization condition (using CHAPS/DDM) [8] was tested periodically throughout the screen to confirm receptor activity. Responses were processed and analyzed using Scrubber 2 (Biologic Software Pty. Ltd., Australia).

CCR5 capture and 2D7 Fab binding using Biacore Flexchip

The 1D4-coated chip was assembled into a continuous-flow microfluidic spotter from Wasatch Microfluidics [9,10]. We captured each of the 96 samples for 30 minutes on the chip. Once the chip was spotted, the flow cell was assembled and docked in the Flexchip instrument. Fab (100 nM) was flowed across the spots for seven minutes, followed by buffer wash of eight minutes.

We used interspot referencing to correct for any instrument drift and then multiplied the responses by 15000 to convert the signal from mdeg to resonance units (RU). Exported response data were processed and analyzed using Scrubber 2 (Biologic Software Pty. Ltd., Australia).

Results

GPCR detergent screening

Figure 1 illustrates the basic steps we developed to screen solubilization conditions for GPCRs in a 96-well-plate format. Step 1 involved a brief sonication of ~6 million cells expressing C9-tagged CCR5 to create a homogeneous suspension. After sonication, the suspended cells were dispensed into a 96-well plate and different detergents were added to the individual wells. The plate was gently rotated for six hours to promote receptor solubilization. After centrifugation to remove any precipitate, the supernatants were transferred and split into two new 96-well plates. One plate of solubilized samples was inserted into the autosampler of the Biacore 2000 and the second was used in the Flexchip analysis.

Screening detergents using Biacore 2000

With the serial-processing Biacore 2000, we tested three detergent conditions within each assay cycle by injecting three differently solubilized CCR5 preparations across individual flow cell surfaces (Fig. 2). In this example, the first detergent effectively solubilized the receptor as we see a significant increase in binding response during the capturing step (green sensorgram). The second detergent failed to solubilize CCR5 or completely blocks binding of the receptor onto the capturing surface (blue sensorgram). The third condition allows for some capture of the receptor (pink sensorgram). After a five-minute washing step the activity of the captured CCR5 was tested using a conformationally sensitive Fab injected at ~1200 seconds. From the binding responses we see that as expected there was no binding of the Fab to the surface which did not capture any receptor (blue sensorgram). We see very little binding response on the third surface, which captured 300 RU of receptor, indicating that this condition may not stabilize the solubilized receptor (pink sensorgram). Finally, we see a significant binding response for the Fab to the receptor captured on the first surface (green sensorgram), indicating that this detergent condition maintains a folded conformation of the receptor at least in terms of Fab binding.

Using the Biacore 2000, in one binding cycle we can quantitate how much receptor was captured and then compare these values with the amount of Fab binding. Another advantage of this capturing method is that the 1D4 surface can be regenerated. This allowed us to automatically screen a large panel of detergent conditions relatively efficiently. Including regeneration and washing steps, just under 24 hours were required to analyze one 96-well plate of conditions.

Figure 3 shows the overlaid binding cycles (separated by flow cell) from all 96 samples of this detergent screen. Many detergents did not solubilize the receptor and several produced solubilized but inactive CCR5. Only a small subset of detergents yielded active solubilized receptor.

The plot in Figure 4 shows report points taken at the end of the wash step versus at the end of the Fab binding step. The conditions that failed to solubilize the receptor (blue and pink data points) cluster at zero. Those that solubilized but inactivated the receptor (orange data) are scattered along the x axis. Interestingly, many of the detergent conditions that yielded good binding signals for the Fab (green and red data) appear to sit on the dashed line. This linearity indicates the activity of the receptor under these conditions is in fact the same: the Fab binding

response intensities are different because they relate to the amount of receptor captured under each condition. Figure 4 demonstrates the benefit of testing both receptor solubilization and activity levels.

Screening detergents using Biacore Flexchip

Using a Wasatch Microfluidics spotting instrument [9,10], we captured the solubilized CCR5 preparations at 96 positions on a 1D4 mAb-coated Flexchip slide. Figure 5 shows an image of the slide's surface after receptor capture. The bright squares represent regions where receptor was deposited. Varying spot intensities represent varying levels of capture. Each ligand spot is well defined, indicating that none of the captured CCR5s spread to neighboring reference spots or contaminated nearby ligand spots.

Figure 6 shows the responses for 2D7 collected across the spotted CCR5 array. In Figure 6A the response profiles from many of the spots show similar kinetics but different intensities. The 2D7 intensity level is a combination of the amount of CCR5 captured as well as its ability to bind the conformationally sensitive Fab. Figure 6B shows report points taken at the end of the Fab injection plotted versus spot position. In this view it is easier to see that a majority of the detergents did not support CCR5 binding activity, which is in fact similar to the results obtained from the Biacore 2000 assay.

Correlation between Biacore 2000 and Flexchip screens

Figure 7 compares the Fab binding levels measured using the Biacore 2000 and Flexchip assays. Maltosides with a C9- to C13-alkyl chain are shown in red and maltosides with shorter and side chains are in pink. The green data points are for three non-maltosides (Cymal-6, Cymal-7, and sucrose monodecanoate) that yielded active solubilized CCR5 (other non-maltosides are shown in blue). Overall the correlation between the two assays is very good, indicated by the data points that line in a diagonal across the plot. Deviations from this diagonal are most likely due to differences in receptor capture levels. In both assays the same thirteen detergents were identified as viable solubilizing agents, all of which were easily discernable from the remaining detergents in the panel. Ten of the best detergents were maltosides. And in both assays, the short- and longest-chain maltosides failed to support activity while three non-maltoside detergents maintained receptor activity. The similarity between these screening results further validates both sensor approaches.

Discussion

With recent breakthroughs in structural approaches to GPCRs [11–13], there is an increased interest in purification approaches for this class of receptor. The first challenge is to identify solubilization conditions that yield high amounts of receptor in a native conformation. Here we demonstrated a method for testing large panels of conditions using the standard 96-well-plate format. With this method, we identified detergents that effectively solubilized receptor while maintaining its activity. We can now test combinations of the most-promising detergents to optimize receptor solubilization and activity further.

We also detailed screening approaches applicable for both serial and array biosensor platforms. The utility of serially processing biosensors is well established [6]. The benefits of these instruments are their ability to measure receptor capture levels and their sensitivity (the ability to detect small molecule binding is particularly important in GPCR studies). But, the serial approach is time sensitive. It requires more time to perform the analysis compared to the array approach and, since the analysis of different receptor preparations are staggered in time, we need to track receptor activity levels throughout the experiment. Also, this approach requires regenerating the capturing surface after each binding cycle. For this CCR5 system, we could

strip the C9-tagged receptor from the 1D4 surface, but we have examined other C9-tagged system for which our regeneration conditions were not successful (data not shown). So, identifying appropriate regeneration conditions can be problematic (or sometimes not possible; for example, this approach cannot be used for biotin-tagged systems captured on standard streptavidin surfaces).

Our approach using the relatively new Flexchip array biosensor worked well for two major reasons. First, we used a continuous-flow spotting device in combination with prototype dextran-coated slides. Traditional pin-spotting methods for array biosensors in the past limited our choice of ligands to pure, highly concentrated solutions that could be dried without significant loss in activity (e.g., peptides, oligonucleotides, and some antibodies) [10]. The continuous-flow spotter can extract the relatively fragile GPCR out of crude supernatant and maintain its micro-environment throughout the spotting process. In addition, we can perform the capturing step for as long as we like, making it possible to concentrate proteins from dilute, as well as crude, solutions. The prototype dextran surfaces were essential to improving capture levels while at the same minimizing nonspecific interactions with the sensor surface. A limitation of the array, however, is that while we can estimate spot densities by looking at the intensity of each spot compared to non-spotted regions, this is not as simple or quantitative as in the serial platforms that directly monitor capture levels.

The array's second advantage is its speed. Ligand spotting and analyte binding can be completed in less than one hour and we can monitor binding at an array of ligand spots simultaneously. No regeneration of the surface is required and all the receptor preparations are solubilized and analyzed at the same time. The Flexchip's simultaneous analysis of all spots means we do not need to account for loss of ligand activity over time. These features are particularly important for the analysis of unstable GPCRs. In addition, the array's rapid sampling allows for flexibility in experimental design. With this approach we can do time-course studies and test combinations of solubilization conditions (e.g., various concentrations of different salts, lipids, and detergents, as well as pH) in a large matrix. But with greater speed comes sacrifice. To date, array biosensors are limited in sensitivity to testing relatively large analytes.

It was important to see that both biosensor assays yielded the same results. The majority of the detergents that maintained CCR5 activity were maltosides with a C9 to C13 alkyl tail. Maltosides having short ($\leq$ C8) or long ($\geq$ C14) alkyl chains; these were ineffective solubilizers. We identified three non-maltosides (sucrose monodecanoate, Cymal-6, and Cymal-7; shown in green) that also yielded good levels of solubilization and binding activity. And the other non-maltoside detergents did not solubilize the receptor and/or failed to maintain its activity.

But the biggest disadvantage of current serial and array biosensors is that both test analyte binding using only one buffer condition at a time. While we solubilized the receptor under 96 different detergent conditions, the binding studies were performed with a single buffer. So, to examine how components of the analysis buffer affect GPCR activity requires testing binding using one analysis buffer, then switching the instrument to another buffer and repeating the experiment. While we identified promising solubilization conditions for CCR5 using established analysis buffer conditions, there may be a more optimal buffer system for this receptor. As array biosensor technology advances, we foresee systems that allow real-time capture and monitoring of activity under different analysis conditions in parallel.

Conclusions

The solubilization method we outlined here provides the means to characterize 96 (or more) conditions in one experiment. While we evaluated the viability of different detergents, this

method can also be used to optimize other solubilization buffer components. Furthermore, we demonstrated the serial and array biosensors each have distinct benefits and limitations for screening GPCR solubilization conditions.

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Abbreviations

BSA	bovine serum albumin
CHAPS	[3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate/N,N-Dimethyl-3-sulfo-N-[3-[[3 α ,5 β ,7 α ,12 α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]propyl]-1-propanaminium hydroxide, inner salt]
CHS	cholesteryl hemisuccinate tris salt
DDM	n-dodecyl- β -D-maltopyranoside
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt)
GPCR	G-protein-coupled receptor
HBS	HEPES-buffered saline
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
OGPS	n-octyl- β -D-glucopyranoside
PEG	polyethylene glycol
RU	resonance unit
SPR	surface plasmon resonance

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Figure 1. Steps involved in screening detergents for GPCR solubilization. (A) Sonicate cell suspension. (B) Combine cell lysate with detergent panel. (C) Mix to solubilize receptor. (D) Centrifuge to remove cell debris. (E) Transfer supernatant to sampling plate. (F) Test for capture on tag-recognizing surface and for binding by conformationally sensitive analyte.

Figure 2.

One capture/binding cycle of screening detergents using Biacore 2000. CCR5 solubilized with one detergent was captured by immobilized 1D4 mAb in flow cell 2 (green), with another detergent in flow cell 3 (blue), and with a third detergent in flow cell 4 (pink). After a 300-s buffer wash, CCR5 capture levels were measured at $t = 1100$ s. At $t = 1180$ s, 2D7 Fab was injected across the captured receptor surfaces, as well as the reference surface (fc1, 1D4 mAb alone), for four minutes, after which 2D7 binding levels were measured and the 1D4 surfaces were regenerated. For the analysis of 96 detergents, this sequence was repeated 32 times.

Figure 3.
Overlaid binding cycles, separated by flow cell, obtained for the panel of detergents using Biacore 2000.

Figure 4.

Correlation between levels of captured CCR5 and 2D7 binding. Data points obtained for maltosides having a alkyl chain length of C9 to C13 are shown in red, pink depicts maltosides with a $\leq C8$ or $\geq C14$ alkyl chain. Non-maltoside detergents that poorly solubilized the receptor are shown in blue; orange depicts non-maltosides that solubilized and inactivated the receptor, green depicts nan-maltosides that yielded active solubilized receptor. The dashed line indicates a linear correlation between the capture and binding levels of the active, solubilized CCR5 preparations.

Figure 5. Image from the Biacore Flexchip of a sensor surface patterned with receptor solubilized using different detergents. Using Wasatch Microfluidics' spotting technology, CCR5 (solubilized using different detergents) was deposited at discrete spots of the 1D4-coated chip surface.

Figure 6. 2D7 Fab binding to the matrix of CCR5 spots shown in Figure 5. (A) Double-referenced responses obtained for 2D7 simultaneously injected across all the different CCR5 preparations. (B) Response intensities at $t = 450$ sec plotted against spot position.

Figure 7. Correlation between Fab binding levels measured using Biacore 2000 and Flexchip. Data points obtained for maltosides having a alkyl chain length of C9 to C13 are shown in red, pink depicts maltosides with a $\leq$ C8 or $\geq$ C14 alkyl chain. Cymal-6, Cymal-7, and sucrose monodecanoate are shown in green, the other non-maltosides in blue.