Integral membrane proteins (IMPs) pose a challenge to study in vitro, as it is difficult to reproduce the membrane-imbedded context of their native state. In this study, we developed a generalized strategy for the assembly of chimeric IMPs within a phospholipid bilayer surface based on a two-step process that first imbeds the insoluble domain of the IMP into a phospholipid layer and then ligates the soluble part of the IMP to the imbedded portion under mild biochemical conditions using a mutant sortase A enzyme. The approach is demonstrated using the transmembrane domain of epidermal growth factor receptor (EGFR) and the soluble extracellular loop of the B-lymphocyte antigen CD20 protein in a POPC tethered bilayer membrane (tBLM). The conditions of the enzymatic reaction were optimized for peptide ligation at the tBLM surface and the role of Ca²⁺ ions in ligation efficiency examined. Additionally, binding of the CD20/EGFR chimera in the context of a membrane environment by the rituximab antibody was measured to assess functionality.

KEYWORDS
CD20, EIS, rituximab, sortase A, SPR, TM domain of epidermal growth factor

1 INTRODUCTION

Characterization of the structure and function of integral membrane proteins (IMPs) comes with major challenges due to the hydrophobicity and subsequent instability of IMPs in aqueous conditions. Investigating IMPs while imbedded in native-like membrane environments is a pragmatic approach to more accurately mimic their function in vivo. A number of approaches for sequestering IMPs within biomimetic lipids have advanced in recent years. In vitro cell-free expression has been used to directly incorporate the expressed IMP into phospholipid layers. Moreover, co-expression of the IMP or transmembrane (TM) domain with detergents, nanodiscs, and in conjunction with artificial membrane scaffolds (soluble lipid vesicles, lipid mesophases, etc), as well as pre-formed tethered bilayer membranes (tBLMs) have all been successfully demonstrated. However, these approaches have their limitations. Detergents used for reconstitution can alter the structure of IMPs and result in significant changes in the protein function. Alternatively, cell-free expression could be used to generate reconstituted IMPs but requires significant refinements in the protocols to ensure protein functionality and insertion into the membrane. To overcome these limitations, here, we have developed and used a new strategy to incorporate IMP into the membrane using a two-step approach. First, the insoluble part of the IMP is inserted into the membrane, and then, the soluble ectodomain of the IMP is ligated to the imbedded TM domain using an enzymatic reaction under mild reaction conditions that will not alter protein structure.
Hydrophobic TM domains of many IMPs predominantly form α-helical structures in the membrane, which, in addition to anchoring the proteins within the cell membrane, play an important role in protein folding, signal transduction, and stabilization of receptor complexes. A number of peptides, derived from TM domains, have been successfully synthesized, imbedded in membranes, and characterized. For example, the TM domain of glycophorin A (GPA), CD4 co-receptor, cystic fibrosis transmembrane conductance regulator (CFTR), and others has been studied. pH-triggered peptides, a relatively new type of TM domain in which a change in pH of the buffer can trigger insertion of the peptide into the membrane, can also be exploited for these purposes. A detailed study of insertion of one of the first pH triggered peptides, pHLIP, was presented by Shu et al and shows a gradual structural transition from unordered structure to helical when pH changes from 7.4 to 5.3.

In our previous work, we developed a method to insert the TM domain of the epidermal growth factor receptor (EGFR) with an N-terminal biotin tag into a POPC tethered bilayer membrane (tBLM). Using tryptophan fluorescence, neutron reflectometry, surface plasmon resonance (SPR), and electrochemical impedance spectroscopy (EIS), it was shown that the EGFR TM inserted in the tBLM forms an α-helical structure and that the biotinylated portion of the peptide is available for streptavidin binding. In principle, this type of TM domain could be synthesized and specifically modified such that it is anchored in a tBLM while having a biochemical or chemical handle of choice exposed for covalent ligation of a solvent exposed, surface domain of an IMP. To demonstrate this concept, here, we show a proof of principle example of chimeric IMP assembly by ligation of the ectodomain of a membrane receptor protein to a TM peptide inserted within a tBLM. We use the same EGFR peptide from the earlier study and the extracellular domain of the CD20 protein to create a CD20/EGFR transmembrane fusion protein inserted in to a tBLM surface.

The CD20 protein is a target for immunotherapy for treatment of B cells malignancies, as it is expressed in more than 80% of B cells lymphomas. The extracellular domain of the protein contains an unstructured loop of about 182 residues and forms oligomers at the cell surface. For immunotherapy, CD20 can be targeted with the monoclonal antibody rituximab (RtxAB). Although RtxAB was approved by the FDA more than 20 years ago and is a widely used therapy, there remain many open questions regarding resistance to the drug and its mechanism of action.

An important consideration in choosing a method to ligate proteins to create chimeric IMPs imbedded in membrane bilayers is that the methods work in mild nondenaturing conditions. A number of native, enzymatic-based ligation strategies based on microbial transglutaminase from Streptomyces mobaraensis, phophopantetheine transpherase, transpeptidase sortase A (SrtA) from Staphylococcus aureus, and enzymes involved in prenylation have recently been developed, which meet this requirement. Among these approaches, SrtA enzyme has been developed into an advanced molecular biology tool that has been used in a number of applications. SrtA specifically ligates a C-terminal LPXTG sequence on one polypeptide chain (where X is any amino acid) to a Gly-Gly (GG) motif on the N-terminus of another polypeptide chain. An SrtA variant, with 140 times the activity of wild type enzyme, has also been engineered through mutation of seven residues in the native enzyme (the so-called “7M SrtA”).

In this work, EGFR peptide was modified to contain two GG amino acids at the N-terminus, and the extracellular loop of the CD20 protein was modified to contain the LPKTG motif on its C-terminus. The 7M SrtA (further “SrtA”) catalyzed ligation reaction was then optimized to achieve formation of a chimeric IMP, functionally inserted in a bilayer phospholipid membrane (tBLM). The binding kinetics of the rituximab antibody to the EGFR/CD20 chimeric protein could then be determined in a membrane-bound context that more closely mimics the in vivo environment.

2 MATERIALS AND METHODS

2.1 Peptide synthesis

Peptides (Table 1) were synthesized by Biomatik, Inc (Wilmington, DE), purified to >95% with high-performance liquid chromatography and molecular weights verified by mass spectroscopy. The N-terminal sequence of EGFR TM peptide (with conserved TM sequence underlined in Table 1) differs from our previous work at the N-terminus: biotin was replaced by the GGHTAQNL motif, which contains two glycines required for the SrtA enzymatic reaction. Soluble K_pep and non-K_pep peptides were used to evaluate SrtA ligation efficiency at the gold substrate surface and represent the soluble part of the EGFR peptide. In the non-K_pep sequence lysines were substituted by random amino acids to remove the strong positive charge created by Lys residues. Both these peptides have glycines at the N-terminus and a cysteine at C-terminus, used to form sulfide bond with gold surface. Peg_pep peptide has polyethylene glycols on N-terminus and a cysteine at C-terminus and was used as a filler molecule to block the bare gold surface between self-assembled K_pep or non-K_pep peptides and prevent nonspecific adsorption.
Two variants of CD20 peptides were synthesized (Table 1): CD20W and CD20H with and without histidine tag. Both have biotins at the N-terminus and disulfide bonds between residues Cys5 and Cys21. Five amino acids at C-terminus were substituted with the LPKTG motif as required for the SrtA ligation.

EGFR peptide was dissolved in 10% (v/v) acetonitrile (AcN) in 50mM Tris-HCl (Tris) buffer (pH 7.4, 150mM NaCl), CD20 peptides in Tris buffer, and soluble peptides in high purity (18 MΩ) water, all with stock concentration of 2mM.

2.2 Reagents for tBLMs planar lipid layers preparations

Powders of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in anhydrous-grade ethanol (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10mM. WC14 thiol (1, 2-di-O-myristyl-3-[ω-mercaptohexa (ethylene oxide) glycerol]) was used as a tethering molecule and was synthesized and purified in our laboratory.27 β-mercaptoethanol (βME) (Sigma-Aldrich) was used as a space-filling component and was distilled before use.

2.3 (7M) Sortase A preparation

The sequence of SrtA (Table 1) was adopted from the work of Guimaraes et al28 and corresponds to what is now referred to as the “7M” mutant due to the seven mutations, five of which improve sortase activity (P94R/D160N/D165A/K190E/K196 T), and two of which confer calcium independence (E105K, E108Q).26 The expression of the protein was followed by the published protocol,28 with one exception: the enzyme was not purify over a gel filtration column, but instead dialyzed away the imidazole using a 10 kDa MWCO membrane. SrtA was tested using a SensoLyte 520 sortase A activity assay kit from AnaSpec, Inc (Fremont, CA). Results of the test are shown in Figure S1 (Supplemental materials section). Measurements were conducted using a Synergy Neo2 instrument (BioTek, Inc Winooski, VT).

Rituximab antibody drug product (RtxAB) was acquired from the pharmacy. The formulation consists of 10 mg/ml Rituximab, 9 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and pH is 6.5 (https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/103705s5311lbl.pdf).

2.4 Phospholipid bilayer formation

Detailed procedures for tBLM formation on the SPR/EIS substrate surface has been described previously.6 Briefly, sapphire substrates (Rubicon Technology, Inc, Bensenville, IL) were covered with 0.5 nm of chromium and 46 nm of gold films, incubated in 0.2mM ethanol solution of WC14/βME (20/80 molar ratio) overnight to form self-assembled monolayer of tethering molecules. After rinsing in ethanol solution, the activated SPR chip was installed in an SPR/EIS cell and a tBLM was formed with the fast solvent exchange method using Tris buffer. Overlayers of POPC molecules were removed from the tBLM surface by incubation in 10% to 20% ethanol solution in water for 10 to 15 minutes. After rinsing with buffer, the tBLM was allowed to rest for 40 to 60 minutes to stabilize the SPR and EIS signals. Insertion of EGFR peptide in the membrane was carried out in Tris buffer with 10% (v/v) acetonitrile (AcN/Tris) by incubation of 10μM EGFR for 60 minutes. Due to the self-assembled nature of the tBLM formation, phospholipid densities may vary and lead to variation in the measured resistance of the membrane. tBLM phospholipid density can be controlled in two ways: (1) reducing the density of tethered molecules by reducing the ratio of WC14 to βME (in this case the ratio was 20 to 80) or (2) applying a

| Name | Sequences |
|------|-----------|
| EGFR | GGHTAQNLKKKLIATGMVGALLLWVVALGIGLFMKMKK-NH₂ |
| K_pep | GGHTAQNLKKKLIATSTSTSTSC |
| non-K_pep | GGHTATSGHDTSTSTSTST |
| Peg_pep | Ac-miniPEG-MiniPEG-TSGSTSSSTC |
| CD20 | Biotin-NYNCEPANPSEKNSFSTQCYLPLKGT |
| CD20H | Biotin-NYNCEPANPSEKNSFSTQCYLPLKGTGHHHHHH |
| 7M SrtA | MQAKPQPKDKSVAGYIEPDADIKEPVYPATREQLNRGSFPAEBERSLDQNISLAGHTDRPNQYQTNLKAARKGSMVYFKVGEHTRKYYKMSRSNNVKPTAVVLDEQKGDOKQILITCDDYNEETGVTETRKFVATVEKLEHHHHHHH |
20% solution of ethanol to the tBLM to solubilize and remove phospholipids from the surface or both. In the experiments, tBLMs with resistance in the range of 20 to 300 KΩ were typically used.

2.5 | Modification of SPR chip with soluble K_pep and non-K_pep peptides

For optimization of SrtA–mediated binding kinetics, soluble peptides were attached to the gold surface of the SPR chip via cysteine at the C-terminus of the peptides. As shown in Figure S2, 0.2mM concentration K_pep (or non-K_pep) in water was adsorbed to the bare gold surface. To block nonspecific adsorption on any bare gold surface between the peptides, Peg_pep peptide terminated with PEG was then adsorbed, followed by adsorption of PEG thiol terminated with OH group. To rinse the surface of nonspecifically bound peptides and PEG thiol, it was treated with 30mM HCl followed by rinsing with 50mM NaOH and finally with rinsing with water and Tris buffer.

2.6 | SPR/EIS measurements

SPR/EIS measurements were conducted simultaneously on the same sample surface employing a home-built SPR instrument and Solartron EIS station (Solartron Analytical, Farnborough, UK), using a saturated silver–silver chloride MI-401F reference electrode (Microelectrodes, Inc, Bedford, NH). The details on the SPR/EIS measurements system and EIS data collection and analysis have been previously described.6

3 | RESULTS AND DISCUSSION

3.1 | Optimization of the (7M) sortase A ligation reaction

The enzymatic reactions with substrates immobilized at the surface do not follow Michaelis-Menten kinetics.29,30 Theoretical models for these reactions have been developed for only the simplest case.31 In our experiments, the SrtA–mediated enzymatic reaction25 involves formation of the complex with protein substrate CD20 containing LPXT motif in the solution, followed by SrtA/CD20 complex adsorption and ligation to the GG motif of the peptide substrate at the surface. In our case, the peptide substrate is the GG-bearing EGFR immobilized at the surface, which results in CD20 ligated to EGFR, followed by release of the enzyme. More over this reaction is reversible26 and can be used to remove the CD20 from the EGFR. Currently, there are no theoretical models for our use case for the SrtA enzyme; therefore, optimization of reaction conditions was conducted empirically.

To simplify optimization of the enzymatic reaction conditions without the complicating factor of the degree of exposure of a peptide imbedded within a tBLM and to increase the concentration of GG ligation motif at the surface, experiments were first performed with peptides directly attached to a gold surface. The soluble K_pep (Table 1) that mimics the N-terminus of the EGFR peptide (part of the peptide above the phospholipid layer) and has a cysteine at the C-terminus to form a sulfur bond with the gold surface was used. Figure 1A shows a schematic of the chemical composition of the SPR chip used for the optimization experiments. Preliminary measurements (Figure S3) showed that RtxAB reversibly bound to CD20 immobilized on the surface allowing optimization experiments to be run using RtxAB binding as an indirect probe of the amount of CD20 attached to surface and hence the efficiency of the ligation reaction. Briefly, the CD20W peptide was ligated to the GG end of the immobilized K_pep peptide by applying 50μM SrtA and 500μM CD20W mixture to the chip surface for 30 minutes (Figure S3). After rinsing with Tris buffer, 1μM of RtxAB (Figure S3) was injected into the SPR/EIS cell. The SPR signal showed significant increase in RtxAB binding in comparison with signal before the addition of SrtA/CD20W. To confirm that the RtxAB was binding to the covalently attached CD20W, and not in a nonspecific manner, 3mM HCl was used to remove nonspecifically adsorbed material from the surface, as indicated by decrease in SPR signal (Figure S3). Repeated application of the RtxAB showed the same signal as before HCl solution rinse, confirming binding to CD20W covalently attached through the GG motif of the peptide. Unfortunately, direct detection of the kinetics of CD20W binding by SPR is masked by nonspecific adsorption of SrtA (Figure S3). Preliminary experiments (Figure S4) also showed the reversible character of the ligation reaction, as expected based on the literature.26 Incubation of the SPR chip with 20μM SrtA for different amounts of time followed by probing with RtxAB showed that SrtA effectively removed covalently bound CD20W from the surface (Figure S4).

In order to push this reversible reaction in the forward (ligation) direction, two parameters must be balanced: an optimal ratio of enzyme to substrate and an optimal total amount of CD20W substrate per exposed GG acceptor on the surface. Figure 1B and Figure S5 show plots of the amount of CD20W ligated to the surface versus molar ratio of enzyme to substrate (CD20W/SrtA). In this experiment, the concentration of SrtA was kept constant at 50μM. From the plot, it is clear
FIGURE 1  Optimization of SrtA–mediated reaction. (A) Schematics of the SPR/EIS chip surface activated with soluble K_pep peptides (shown in black). PEG_pep peptide terminated with PEG (shown in gray) and PEG-OH (marked OH) thiol were used to block nonspecific adsorption to bare gold surface. The CD20 peptide will bind through LPKTG motif to the Gly-Gly N-terminus end of K_pep peptide. SPR data on the chip surface preparation is shown in Figure S2. (B) Dependence of CD20 binding to the K_pep peptide versus molar ratio of SrtA to CD20 (concentration of SrtA was kept constant at 50 μM). (C) Dependence of the CD20 binding to K_pep peptide versus SrtA concentration at constant molar ratio of SrtA/CD20 (1/10). (D) Dependence of the rate of the reverse SrtA enzymatic reaction (amount of CD20 peptide removed from the chip surface in 3 minutes) versus SrtA concentration. EIS, electrochemical impedance spectroscopy; SPR, surface plasmon resonance; SrtA, sortase A

the ligation efficiency increases linearly with increasing CD20W (substrate) concentration up to 500μM. Figure 1C and Figure S6 show the dependence of CD20W ligation versus SrtA concentration keeping the molar ratio of SrtA/CD20 constant at 1 to 10. Under these conditions, binding of CD20W increases with increase of total concentration of SrtA/CD20W mixture.

To check the influence of reversibility of the SrtA enzymatic reaction, we measured the rate of the enzymatic reaction. Different concentrations of SrtA were applied to CD20W bound to the surface and incubated for 3 minutes. Subsequent measurement of RtxAB binding showed how much CD20W was removed from the surface (Figure 1D and Figure S7). For these measurements, the CD20W surface concentration was kept constant for every SrtA injection. As we can see (Figure 1D), the rate of the reverse reaction slows down with the increase in SrtA concentration and above 100μM does not changes significantly. This is most likely a consequence of SrtA binding to the surface attached CD20W. Binding kinetics and diffusion at the surface may differ significantly from the bulk solution.

Another dimension to the complexity of the SrtA–mediated reaction at the interface is the effect of the charge of the chemical groups bound to the surface. In accordance with the literature, in which the 7M SrtA enzyme is not Ca2+ ion-dependent, in our experiments with the K_pep peptide, we did not see any difference in ligation efficiency as a function of CaCl2 concentration (data not shown). However, when four lysine residues in the K_pep peptide were replaced to amino acids with neutral or negative charge (non-K_pep peptide, Table 1), the results changed dramatically. As shown in Figure 2 and Figure S8, there are significant differences in interaction of SrtA/CD20W solution with the non-K_pep (no lysine) peptide at gold surface when the SPR chip is treated with 50mM CaCl2 solution before SrtA/CD20W injection.
FIGURE 2  Dependence of SrtA ligation reaction on CaCl₂ on a surface modified with non-K_pep peptide. K_pep was replaced with non-K_pep, which does not contain positively charged lysines, in the same experimental setup as in Figure 1A. Without treatment of the surface with 50mM CaCl₂ for 15 minutes, the amount of nonspecifically bound SrtA/CD20 increases, as judged by the smaller amount of RtxAB seen to bind CD20 at the surface. SPR, surface plasmon resonance; SrtA, sortase A.

In this case, a significantly smaller adsorption signal for SrtA/CD20W is detected in comparison with the amount of SrtA/CD20W adsorbed without CaCl₂ treatment (Figure 2). However, with calcium chloride treatment more CD20W is covalently attached to the surface as shown by RtxAB binding when compared with the no CaCl₂ injection (Figure 2). Thus, most of the SrtA/CD20W signal increase without CaCl₂ injection is due to nonspecific adsorption that is lost with injection of 3mM HCl solution to remove nonspecifically adsorbed SrtA/CD20W complex (Figure S8). This result suggests that the positive charge at the end of the peptide (K_pep has a net charge of +4, versus non-K_pep that has a net charge of -1) plays significant role in the ligation reaction. Most likely the positively charged surface allows for a more preferable orientation of the SrtA (SrtA has net charge -1) to conduct the ligation reaction while the negatively charged surface increases nonspecific adsorption due to charge-charge interaction but does not result in a preferable orientation for ligation to occur. Additionally, the CaCl₂ effect was only observed with the negatively charged peptide and not with the K_pep (positively charged) peptide. These ligation optimization experiments show that mechanism of CD20W covalent attachment to GG at the surface involves the direct and reversible SrtA reaction in addition to adsorption of the SrtA/CD20 complex to the surface. Taking into consideration, these data and considering a practical timeframe and economical costs of the ligation reaction, the following optimal reaction conditions were chosen: 30 minutes incubation, a ratio of 50μM SrtA to 500μM CD20 mixture and a K_pep modified surface (Figure 3).

As the controls, Figure 3 also shows the SPR response measured when RtxAB is injected before (marked 1, Figure 3) and after the ligation reaction (marked 2) indicating a more than 10-fold increase in RtxAB binding post-ligation of the CD20 ligand. While RtxAB binds reversibly to CD20, streptavidin binds irreversibly to the biotin at N-terminus of CD20 with the SPR response more than 2 times larger than that for the antibody, likely due to significant differences in binding constants, stoichiometry, or availability of epitope. Binding of RtxAB after streptavidin binding (marked 3, Figure 3) is dampened, most likely due to a steric hindrance preventing the accessibility of the RtxAB to the CD20 binding. Post-streptavidin binding, the surface is also unable to be regenerated by another application of SrtA, possibly for the same reasons.

FIGURE 3  The optimized ligation at the surface, as shown in Figure 1A, can also be traced by the following SPR sensorgram. As a control, before starting the ligation, 1μM RtxAB was injected in to the cell for 5 minutes (marked 1). The ligation process starts after injection of 50μM SrtA/500μM CD20 mixture (next arrow shows starting point of the injection) and continues for 60 minutes. After rinsing with buffer to remove SrtA/CD20 solution, to demonstrate that ligation was successful, 1μM RtxAB is injected to the cell (marked 2) for 5 minutes. Following the buffer rinse, the response is reduced to baseline due to the reversibility of the RtxAB binding. Therefore, for additional confirmation by showing binding to biotin at the C-terminus of the CD20 protein, 1μM of streptavidin was injected for 5 minutes in the cell. Finally, 1μM RtxAB injection (marked 3) was applied after rinsing of streptavidin from the cell. SPR, surface plasmon resonance; SrtA, sortase A.
There is no difference in the ligation process for CD20W and CD20H we found experimentally, and the two proteins could be used interchangeably in the surface experiments (data not shown).

Ligation with SrtA enzyme creates a covalent bond but remarkably, by changing the conditions, the reaction is fully reversible. This property may be very useful in many biochemical applications. However, ligation with SrtA comes at a high price tag: the requirement for high concentrations of the ligand. The 7M SrtA that we used in our work significantly improves ligation efficacy in comparison with wild type.\(^{26}\) However, even with the improved (140×) efficiency, large concentrations of enzyme were necessary for the ligation due to the small binding affinity to the GG motif at the surface and the need to use a large molar ratio of enzyme/ligand. It is possible that Ca\(^{2+}\) ions may play a regulatory role in forward and reverse ligation process of SrtA and, if this is a case, it can be exploited for future improvements of ligation efficiency. As it is shown, the positive charge on the N-terminus of the substrate with GG motif also improves ligation efficiency.

### 3.2 Assembly of chimeric transmembrane CD20/EGFR protein

The tethered phospholipid bilayer (Figure 4A) was formed by the solvent exchange technique as described in the Methods section. The final resistance \((R_m)\) measured by EIS of the tBLM after formation and before EGFR peptide insertion at pH 7.4 Tris buffer was 161.2 K\(\Omega\), an indication of a well-formed bilayer. Difference in EIS spectra with and without phospholipid membrane and the electrical model used for tBLM resistance \(R_m\) calculations are shown in Figure S9.

For EGFR insertion, the Tris buffer was exchanged to 10% acetonitrile/Tris (AcN/Tris) buffer followed by injection of a solution of EGFR in the same buffer to the SPR/EIS cell and incubated for 60 minutes (Figure S10A). As in our previous work, after rinsing with AcN/Tris buffer and switching back to Tris, no difference in SPR signal was observed—most likely due to fluidity of the membrane that keeps the same density during gradual peptide insertion into tBLM. However, the resistance of tBLM after EGFR insertion dropped dramatically to 23.6 K\(\Omega\) at pH 7.4 (Figure 4B), indicating changes in the density of phospholipid bilayer and interaction between head groups of phospholipids and peptide.

For the second step in reconstruction of the chimeric IMP, we used the optimal SrtA ligation conditions, as described in the previous section. The fusion EGFR/CD20 transmembrane protein indeed formed, as demonstrated by efficient binding of the RtxAB (Figure 5A black curve). The gray curve in Figure 5A shows a control, when a mixture of the SrtA and CD20 was injected to a cell with the tBLM surface without inserted EGFR peptide. In this case, all SPR signal increase after injection and before rinsing with buffer can be attributed to a change in the bulk refractive index due to the very high concentration of the injected protein mixture. After rinsing of the mixture from the cell, no significant increase in the SPR signal was detected, strongly suggesting negligible binding of SrtA or CD20 to tBLM surface. Injection of 1 \(\mu\)M of RtxAB (marked 1, Figure 5A, gray curve) also shows a very small, less than on our model surface (Figure 3, marked 1), nonspecific reversible binding. In contrast, when EGFR is inserted to the tBLM (Figure 5A, black curve), the SPR signal increase is much larger upon SrtA/CD20 complex injection, and in comparison to the bulk refractive index change observed in the control (Figure 5A, gray curve), showing complex binding. After rinsing, the SPR signal remains strong and correlates well with data shown in Figure 3.

**FIGURE 4**: Assembly of chimeric CD20/EGFR transmembrane protein in biomimetic tethered bilayer membrane and it interaction with rituximab antibody. (A) Cartoon schematic of tBLM at gold surface with inserted EGFR peptide (red coil) with covalently attached CD20 and bound RtxAB. (B) Difference in tBLM electrical resistance before EGFR peptide insertion into the tBLM and after. EGFR, epidermal growth factor receptor; tBLM, tethered bilayer membrane.
FIGURE 5  SPR data on formation of chimeric CD20/EGFR transmembrane protein inserted into the tBLM. (A) SPR data on ligation of the EGFR peptide at tBLM surface with SrtA enzyme and CD20 peptide. Gray curve shows control, when no EGFR peptide is in tBLM. Black curve shows response with formation of the chimeric CD20/EGFR IMP at tBLM with inserted EGFR peptide. Incubation time of the mixture of SrtA/CD20 in both cases was 40 minutes, followed with Tris buffer rinse and RtxAB and streptavidin injections as in Figure 3. (B) Binding of rituximab Ab to the chimeric CD20/EGFR IMP. Black dots show RtxAB adsorption versus concentration at steady state. Data are corrected for nonspecific RtxAB adsorption. Insert shows dependence of the RtxAB adsorption versus time at different concentrations in the range from 0.06 μM to 3 μM. Protein injection time was 5 minutes, followed with Tris buffer rise. EGFR, epidermal growth factor receptor; SPR, surface plasmon resonance; SrtA, sortase A; tBLM, tethered bilayer membrane

As in the model surface, the observed binding of RtxAB and streptavidin to the tBLM surface also provides strong confirmation of fusion CD20/EGFR IMP formation. The almost two times smaller SPR signal (17.6 vs 8.7 pixels) of RtxAB shown in Figure 5A in comparison to Figure 3 is likely due to the smaller surface concentration of the EGFR peptide inserted into tBLM layer. The streptavidin SPR signal is also smaller (47.9 vs 17.6 pixels) on tBLM surface for the same reason; however, the ratio of streptavidin to RtxAB (2.7 vs 2.0) is larger in the case of model surface in Figure 3. This observation may be the result of the difference in the protein sizes that can lead to the differences in surface area that occupy antibody and streptavidin.

To determine the dissociation constant of the RtxAB antibody from the fusion CD20/EGFR IMP, the kinetics of RtxAB binding versus concentration were measured (SPR trace vs time is shown as an insert in Figure 5B). Steady-state binding of RtxAB to the fusion CD20/EGFR IMP (Figure 5B) fit to a single exponent model suggesting 1:1 binding model and a dissociation constant $K_D$ of $310 \pm 70 \text{ nM}$ (error was calculated from 3 independent replicates). The data plotted in Figure 5B were corrected for nonspecific RtxAB interaction with tBLM before fitting (nonspecific binding of RtxAB at tBLM versus concentration is shown in Figure S10B). Attempts to fit RtxAB adsorption kinetic data directly to the SPR response vs time (Figure 5B insert) to single or double exponential model in order to obtain rate of adsorption and desorption were unsuccessful, most likely due to diffusion limited kinetics. Taking into consideration the high molecular weight of RtxAB (~145 kDa) and fast adsorption and desorption rates of RtxAB (Figure 5B insert), the diffusion limitation is a reasonable assumption. The use in our experiments of a nonflow cell limited our ability to control flow rates, which could have been used to reduce the effect of diffusion limited kinetics.

The measured dissociation constant $K_D$ of RtxAB to the EGFR/CD20 fusion protein is significantly (two orders of magnitude) weaker than that measured using affinity assays based on radioactive $I^{195}$ at the surface of the Raji cells (4.2 nM)$^{15}$ and other sources (8 nM) (https://www.accessdata.fda.gov/drugsatfda_docs/label/2010/103705s5311lbl.pdf rituximab drug description). On the other hand, the $K_D$ determined here is in better agreement with data obtained on the same Raji cells measured using a quartz microbalance technique (160 nM).$^{14}$ The fast dissociation rates measured here also contradict previous data,$^{15}$ which describe a slower dissociation rate. Taking into consideration the complex mechanism proposed$^{12}$ for RtxAB binding, many contributions may underlie the difference in measured dissociation constant. It has been suggested that RtxAB may bind to tetramers of CD20 protein or that RtxAB itself may be involved in formation of the tetramers. The $F_C$ domain$^{16}$ of RtxAB is also involved in binding to the cell and conformation of the CD20 loop may also play role in the binding.

In our experiments, CD20 has only one RtxAB epitope: the ANPS motif. There are suggestions$^{12}$ that part of sequence YCYSI near the cysteine-mediated disulfide bond contributes to the binding of RtxAB through conformational stabilization. In our chimeric IMP, this part of the sequence is replaced by the LPKTG motif for use in the SrtA ligation.
While the results of our studies raise interesting new questions about the binding of RtxAB to CD20, overall, the data shows the potential utility of engineered fusion IMPs for studying the interactions of these proteins in the context of native-like model membrane environments. By changing the sequence and/or structure of the CD20 ligated to the EGFR peptide, one could further interrogate the different contributions to RtxAB binding. For example, the influence of the disulfide bond in the CD20 loop on the RtxAB binding was probed by simply applying a 20mM solution of the TCEP HCl to reduce the disulfide bond, and interestingly, no difference in binding were observed (data not shown).

4 | CONCLUSION

It is well known that proximity to the phospholipid layer, composition of phospholipid, and lateral interaction of IMPs at phospholipids surface may significantly alter properties and function of these proteins. In this work, we have integrated biomimetic tBLMs, with insertion of the TM domain of an IMP into the tBLM and fusion of the soluble domain of an IMP to the TM domain via an enzyme-mediated ligation reaction as a general strategy for assembling functional chimeric IMPs at phospholipids surface. The strategy involves two steps carried out under different chemical/biochemical conditions: one suitable for insertion of insoluble part of the IMP to phospholipid layer and another for attaching soluble part of IMP to TM to form hybrid protein under mild biochemical conditions. In the context of SPR as well as other methods for measurement of binding, this strategy for chimeric IMP assembly can be adopted for interrogation of the structure and function of IMPs and applied in the context of drug screening. Additionally, with improved methods of enzymatic reaction and using freestanding phospholipid layers, it could be feasible to modify this approach to enable modeling of signal transduction through a membrane. Lastly, it may apply for functionalizing lipid bilayers with IMPs not only in the framework of planar phospholipid bilayers (tBLMs), as shown here, but also in other model membrane systems such as liposomes, nanodiscs, vesicles, and bicelles.

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**SUPPORTING INFORMATION**

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