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ACS APPLIED MATERIALS & INTERFACES

Review

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Nanoyeast and Other Cell Envelope Compositions for Protein Studies and Biosensor Applications

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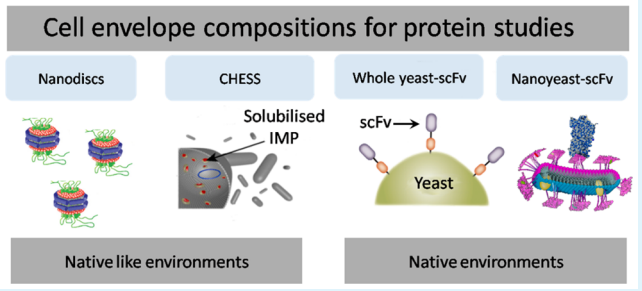
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ABSTRACT: Rapid progress in disease biomarker discovery has increased the need for robust detection technologies. In the past several years, the designs of many immunoaffinity reagents have focused on lowering costs and improving specificity while also promoting stability. Antibody fragments (scFvs) have long been displayed on the surface of yeast and phage libraries for selection; however, the stable production of such fragments presents challenges that hamper their widespread use in diagnostics. Membrane and cell wall proteins similarly suffer from stability problems when solubilized from their native environment. Recently, cell envelope compositions that maintain membrane proteins in native or native-like lipid environment to improve their stability have been developed. This cell envelope composition approach has now been adapted toward stabilizing antibody fragments by retaining their native cell wall environment. A new class of immunoaffinity reagents has been developed that maintains antibody fragment attachment to yeast cell wall. Herein, we review recent strategies that incorporate cell wall fragments with functional scFvs, which are designed for easy production while maintaining specificity and stability when in use with simple detection platforms. These cell wall based antibody fragments are globular in structure, and heterogeneous in size, with fragments ranging from tens to hundreds of nanometers in size. These fragments appear to retain activity once immobilized onto biosensor surfaces for the specific and sensitive detection of pathogen antigens. They can be quickly and economically generated from a yeast display library and stored lyophilized, at room temperature, for up to a year with little effect on stability. This new format of scFvs provides stability, in a simple and low-cost manner toward the use of scFvs in biosensor applications. The production and "panning" of such antibody cell wall composites are also extremely facile, enabling the rapid adoption of stable and inexpensive affinity reagents for emerging infectious threats.



KEYWORDS: *cell envelope composition, affinity reagent, nanoyeast, biomaterial, nanomaterial, immunosensor, biosensor*

1. INTRODUCTION

Proteins are widely used as biomarker targets for medicine,[1] as such protein target characterization and affinity binder production for proteomic immunosensors[2−4] are the focus of extensive research and development. Proteins and antibodies are naturally produced within a biological environment; however, many biotechnology applications require these biomolecules to be solubilized for further study and use. This is problematic because many of these biomolecules often lose stability (denatures by losing its quaternary, tertiary, and secondary structure) once introduced into a foreign (non-native) environment. Membrane-bound proteins in particular have been widely exploited as druggable targets[5] but are difficult to study as solubilized targets due to protein conformation changes in the absence of a stabilizing lipid or cell wall environment. Similarly, synthetic recombinant antibodies produced in eukaryotic or prokaryotic production systems commonly lose stability, especially when they originated in a display library. Many strategies have been developed to overcome these stability issues. Particularly promising are cell envelope compositions to stabilize proteins and recombinant antibodies in native or native-like environments.

Recombinant antibody fragments are a promising class of protein capture reagents which are poised to complement or

Received: July 27, 2016
Accepted: October 20, 2016
Published: October 20, 2016

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DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

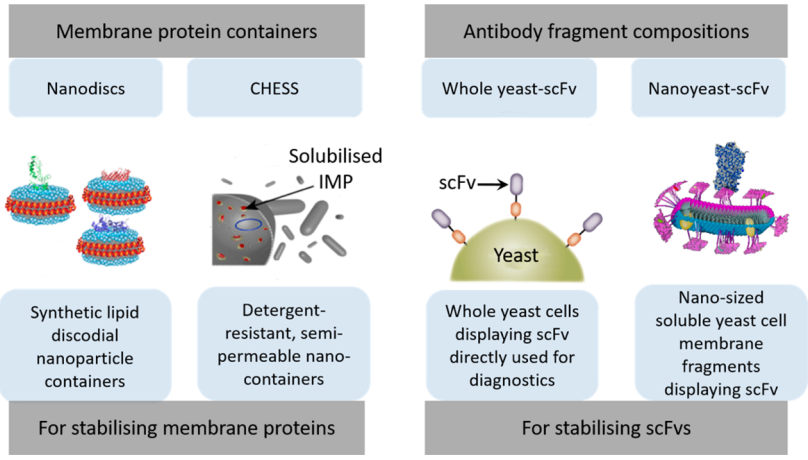


Figure 1. Advances in cell envelope compositions for protein studies. Schematic representation of the development of cell envelope compositions for the stabilization of proteins. Nanodiscs: discoidal nanoparticles with a membrane protein assembled with phospholipids and surrounded by membrane scaffold proteins. Reprinted with permission from ref 32. Copyright 2013 Springer Science+Business Media. Cellular high-throughput encapsulation, solubilization, and screening (CHESS): integral membrane proteins (IMP) encapsulated inside semipermeable nanocapsules allowing for proteins to be solubilized in situ while maintaining the genetic information on the protein. Reprinted with permission from ref 23. Copyright 2013 Elsevier. Whole yeast−scFv: scFv displayed on yeast cell surfaces are kept attached to the cell wall during immunoassays, hence maintaining the environment for which they were selected. Reprinted with permission from ref 30. Copyright 2015 Elsevier. Nanoyeast−scFv: scFvs remain attached to nanosized fragments of the yeast cell wall, producing solubilized immunoaffinity reagents. Adapted with permission from ref 28. Copyright 2015 American Chemical Society.

replace complete, full-length monoclonal antibodies (mAbs) in immunosensors.[2−4] These fragments can show identical specificity toward target antigens as their parent, full-length mAbs. They also have the added flexibility to engineer the fragment antigen binding site, which allows custom production of reagents with the most sought after affinity traits. Furthermore, antibody fragments can be rapidly isolated from libraries of antibody fragment genes using various display technologies. They are renewable and can be produced in eukaryotic or prokaryotic production systems followed by scale-up manufacture to reduce production costs.[6,7] More recently we developed an antibody library biopanning method that utilized whole cells during selections. This ensures the recombinant membrane-bound proteins maintain their native conformation during antibody selections.[8]

One of the most common types of antibody fragments are single-chain variable fragments (scFvs), which are recombinant polypeptides that are composed of a light-chain variable (V_L) domain connected by a flexible hydrophilic peptide to a heavy-chain variable (V_H) domain.[9,10] These 30 kDa monovalent proteins possess comparable specificity and sensitivity to parent mAbs and, due to a lack of a constant domain (Fc) region, are capable of superior performance as imaging and diagnostic agents. Furthermore, the production pipeline of recombinant immunoaffinity reagents could potentially reduce some problems that hamper traditional full-length mAb manufacture,[2,3] such as batch to batch variability and a slow drift in the quality of immunoaffinity reagents over time.[2,11]

Despite these advantages, many solubly expressed scFvs suffer from stability problems.[12−14] This necessitates new designs and strategies to produce stable protein capture agents.[12,15−17] Membrane and cell wall proteins similarly suffer from stability problems once solubilized from their native membrane environment. Biological nanocontainers and other strategies have been developed to stabilize membrane proteins by maintaining a native or native-like lipid environment during protein studies.[18−23] This approach of stabilizing surface proteins in a native environment has been adapted to stabilize recombinant antibody fragments from yeast display libraries.[24−30] ScFvs are stabilized by maintaining the native cell wall environment of the yeast display library from which they were selected. The yeast cell wall is comprised of a semirigid polysaccharide/lipid/protein matrix, which provides structure and rigidity to the cell, protection against physical stress, and a scaffold for surface proteins.[31] Although there are many differences between cell membranes and yeast cell walls, both cell envelope approaches utilize the principle of stabilizing molecules in native or native-like environments to avoid issues with solubilization that can plague proteins and antibodies during protein studies.

Herein, we highlight biological compositions which maintain membrane protein and scFv stability by retaining native or native-like environments, which are designed for easy production while maintaining specificity and stability when in use with simple detection platforms (Figure 1).

2. MEMBRANE PROTEINS AND OTHER CELL-SURFACE PROTEINS

Membrane proteins are utilized in research, diagnostics, and industry. They are widely exploited as drug targets, and there are numerous FDA-approved and experimental small molecule drugs and protein biologics that bind membrane proteins.[33] mAbs are the largest class of protein biologic drugs for indications including cancer and inflammatory diseases.[34,35] Although there are several methodologies that utilize recombinant DNA technology (i.e., hybridoma technology, antibody display technologies) to produce/select mAbs that bind soluble proteins,[6,36,37] generation of useful mAbs that bind surface proteins using display technologies is much more challenging. Surface proteins generally have extensive hydrophobic domains embedded in lipid bilayers and/or polysaccharide cell walls. Removal from these environments renders surface proteins poorly soluble and prone to aggregation, misfolding, and denaturation once introduced into a purely aqueous environment.[38] Solubilization of some surface proteins, especially membrane

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

Table 1. Comparison of Cell Envelope Technologies for Membrane Protein Studies

composition	advantages	disadvantages
(a) nanodiscs	can be immobilized onto surfaces for integration of membrane proteins and soluble molecules	requires correct stoichiometry of phospholipids, MSP and target protein to avoid aggregation and nondiscoidal structures; requires MP to be presolubilized in detergent before insertion into the lipid nanoparticle
(b) styrene maleic acid lipid particles (SMALP)	Does not require the membrane protein to be presolubilized in detergent; encapsulated bilayer retains many of the physical properties of the parent membrane, including the lipid mixture, structural organization, and phase behavior	proteins in excess of ~400 kDa unlikely to fit; SMALP formation requires pH 6.5 or above, and therefore downstream experiments must maintain this pH to maintain SMALP structure
(c) lentivirus	sub-100 nm particles likely amenable to various applications	so far only demonstrated for GPCRs; applications limited to SPR for kinetic studies at this stage
(d) liposomes	act as supports for membrane proteins in biosensor applications; permit protein flexibility and movement while avoiding direct exposure of the proteins to the surface; soft, deformable, biodegradable, and functionally integratable	reconstitution of protein typically a long and labor intensive and unpredictable process; incorporating proteins in an in vivo-like configuration while maintaining functionality is challenging; most methods use simple single heterogeneous membrane structures, with the creation of more complex native-like structures being more difficult to achieve; poor stability
(e) cellular high-throughput encapsulation, solubilization and screening (CHESS)	rapid and high-throughput method for directed evolution of stabilized soluble proteins from a diverse library of millions; physical encapsulation of protein provides protection against detergents during membrane solubilization	to prevent diffusion of proteins out of the nanocontainer, proteins require a link to a fusion molecule so its molecular weight is greater than 70 kDa; ideal range pH 6–9, with potential aggregation at low pHs and dissolution of nanocapsules at high pHs

proteins, can be achieved using detergents. However, detergents can deactivate proteins by denaturing their structure and can be difficult to remove if required.[39] The soluble, extracellular domain of a protein may be expressed and is generally useful; however, there may be changes to the conformation that rely on immobilization within a lipid bilayer or cell wall structure. Whole, intact cells overexpressing the receptor-of-interest can be used but various negative biopanning strategies need to be employed to reduce nonspecific binding.

2.1. Cell Envelope Compositions To Study Membrane Proteins. One strategy to study and utilize membrane proteins while maintaining their native state is to create artificial cell envelope compositions that mimic the native, lipid bilayer environment of membranes. Several examples of manipulating lipid bilayers to stabilize membrane proteins are detailed below and in Table 1.

2.1.1. Nanodiscs. One approach to creating artificial cell envelope compositions is the use of nanodiscs, which are discoidal nanoparticles formed by synthetic lipids and surrounded by a belt consisting of amphipathic domains and α-helical proteins called membrane scaffold proteins (MSPs).[18,19,40−42] The target membrane protein is transiently solubilized with a detergent in the presence of phospholipids and an encircling MSP. When the detergent is removed, the target membrane protein simultaneously assembles with the phospholipids into a discoidal bilayer, with the size controlled by the length of the MSP (Figure 2). The standard method for self-assembling an MP into a nanodisc is shown in Figure 2, route 1: after detergent solubilization and purification, the target MP (green) is mixed with the membrane scaffold protein (MSP, blue) and lipids at the correct stoichiometry, followed by detergent removal through incubation with hydrophobic beads. Often, however, the MP is not stable in detergent for the extended times needed for purification. Alternatively (Figure 2, route 2), the starting membrane or tissue can be directly solubilized with excess lipid and scaffold protein and rapid detergent removal. This results in the placement of the target MP (green), together with other MPs (gray) in the tissue, into the nanodisc. Subsequent purification, often with an affinity tag, is performed, and the target is stabilized in the nanodisc environment.

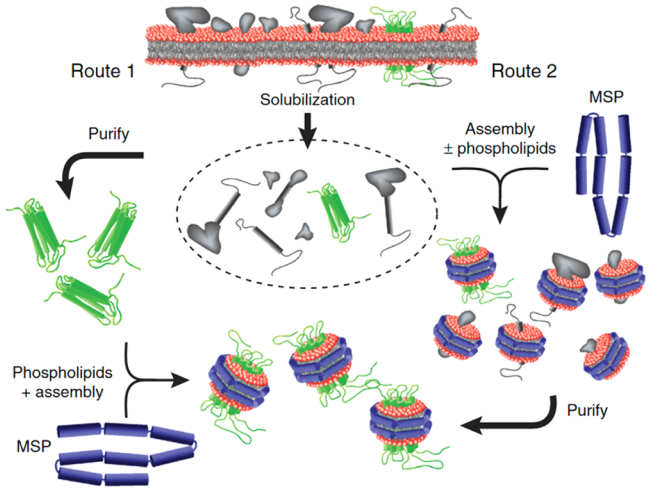


Figure 2. Assembling MPs into nanodiscs. Two different methods (routes 1 and 2) have been developed for the formation of MP into nanodiscs. Route 1 is the traditional method where the target MP (green) is mixed with the MSP. In route 2, the starting membrane is directly solubilized with phospholipids and scaffold protein, which can be purified based on the presence of target MP. Adapted with permission from ref 42. Copyright 2016 Nature Publishing Group.

This latter route can also be used to generate a soluble MP library that faithfully represents the MPs in the starting tissue. The resulting nanodisc keeps the proteins in solution, providing stability by retaining these membrane proteins a native-like phospholipid bilayer environment. Nanodiscs can be immobilized onto surfaces, allowing for kinetic studies and drug binding determination to be carried out between soluble molecules and incorporate membrane proteins.[43,44] Phage display selection can also be performed using nanodisc-bound proteins as targets, to select for high-quality synthetic reagents that bind membrane proteins in native-like lipid environments.[19] Recently, incorporating therapeutic antibodies into nanodiscs was also proposed.[45] A critical factor to successfully forming a nanodisc relies on ensuring the correct stoichiometry of phospholipids, MSP, and target protein.[46] Incorrect stoichiometries

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

of lipids, MSP, and target protein results in aggregates and varying nondiscoidal structures, which are difficult to separate and characterize and to isolate the desired nanolipoprotein particles. The nanodisc method also requires the membrane protein to be assembled from the detergent-solubilized state. Therefore, this method will not work if the target protein is already in an aggregated soluble state—where the protein is already in aqueous solution but likely inactive.

2.1.2. SMALPs. Styrene maleic acid lipid particles (SMALPs) allow membrane proteins to remain stabilized by use of a simple organic polymer (SMA copolymer) that is used to directly extract proteins from membranes into lipid nanoparticles called SMALPs.[47,48] SMALPs contain a central lipid bilayer supported by an outer annulus of the SMA polymer. This approach of extracting membrane proteins and then placing them into a native lipid environment is similar to the nanodisc method detailed above; however, unlike nanodiscs, the SMALP approach does not require the membrane protein to be presolubilized in detergent before insertion into the lipid nanoparticle. The SMALP structure is stabilized by the intercalation of hydrophobic styrene groups between the acyl chains of the lipid bilayer, whereas the hydrophilic maleic acid groups are presented to the solvent. The encapsulated bilayer retains many of the physical properties of the parent membrane, including the lipid mixture[49] and structural organization and phase behavior.[50] Although this method is effective in preserving membrane proteins with a native lipid envelope, there are some limitations need to be taken into account. The disc shaped SMALP has a maximum nominal diameter of 15 nm, which corresponds to a molecular mass of less than ∼400 kDa.[50] Therefore, proteins that are too large to fit within this limit are unlikely to be solubilized. The formation of SMALP also requires a pH above 6.5, which means downstream experiments also need to be carried out at above pH 6.5 to maintain the SMALP structure. The SMA polymer is an effective chelator of divalent cations such as Mg^{2+} and Ca^{2+}, with the chelate also being insoluble.[48] As such, downstream experiments that require high concentrations of divalent cations (>5 nM) could disrupt the SMALP. Divalent cations can be found in membrane proteins that bind nucelotides such as ABC transporters and ATPases. However, experiments could be adjusted with lower concentrations of nucelotides to preserve SMALP integrity while still maintaining native levels of membrane protein activity to work around this limitation.

2.1.3. Lentivirus. Lentivirus particles have been used to present cell membrane proteins on their surface to allow for ligand interaction studies in optical biosensors using conditions that resemble native in vivo environments.[20,51,52] During budding from the cell surface, viruses pull away membrane fragments bearing proteins in their lipid environment. The authors took advantage of this process to present G-protein-couple receptor (GPCR) proteins on the surface of lentiviral particles. GPCR proteins were found to incorporate into virions that were easily purified and attached to a biosensor surface. This method eliminated the need for detergents to solubilize these GPCR proteins, thus allowing for stable environments to conduct kinetic studies. These studies showed that GPCRs could be stabilized on lentiviral surfaces; however, additional experiments are required to demonstrate the universality of this approach toward displaying and stabilizing other membrane proteins for protein interaction and capture studies. Furthermore, lentiviral particles applications have so far been limited to kinetic studies using surface plasmon resonance (SPR) as a readout platform. For lentiviral particles to be positioned as a universal membrane protein stabilization platform, proof of compatibility with other biosensor platforms needs to be conducted. Given that leniviral particles are typically sub-100 nm, it is likely they should be amenable in a wide range of biosensor platforms.

2.1.4. Liposomes. Immobilization into liposomes has also shown promise as a method to stabilize membrane proteins in a native-like environment, which maintains their functionality.[21,53−55] Liposomes are spherical nanoparticles with unilamellar or multilamellar structures that separate and encapsulate an aqueous interior from bulk aqueous solvent. The lamellae of liposomes are composed of a bilayer of lipids with a hydrophobic midplane to separate the two aqueous volumes. Interaction of the lipid with membrane proteins has been shown to promote proper orientation, which influences protein function.[22] In this method, membrane-bound proteins were purified from their native membrane environment and then further reconstituted into liposomes by comicellization of the

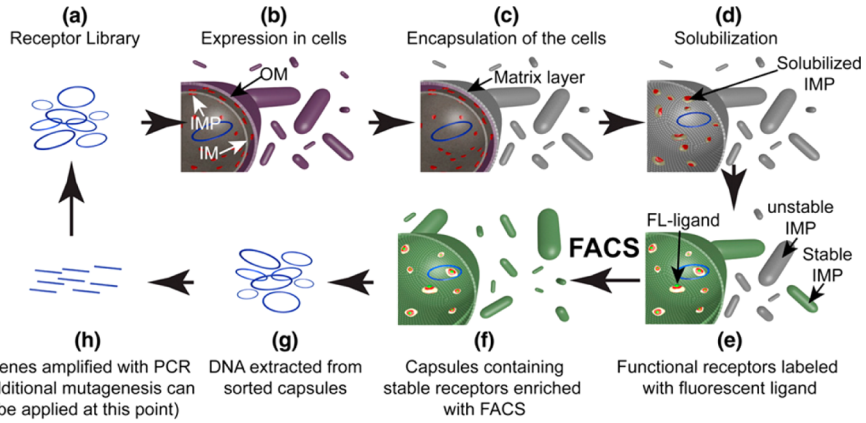


Figure 3. Schematic representation of the CHESS method. Library of receptor mutants (a) is transformed and expressed in the inner membrane (IM) of *E. coli* (b). Cells are encapsulated (c) and the cell membrane is permeabilized with detergent (d), leading to solubilization of the receptor. The encapsulation layer serves as a semipermeable barrier, retaining the solubilized receptor and its encoding plasmid within the capsule, where it can bind to functional receptor molecules (e). Capsules containing detergent-stable GPCR mutants are more fluorescent and be sorted from the population using FACS (f). Genetic material is recovered from the sorted capsules (g) and used to either identify desired receptor mutants or serve as a template for further rounds of mutation or selection (h). Figure and caption reprinted with permission from ref 23. Copyright 2013 Elsevier.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

purified membrane protein in excess of phospholipids and detergents to form a solution of lipid/protein/detergent or lipid/detergent micelles.[22] Dialysis, dilution, or adsorption are common reconstitution methods, with these methods all working by reducing the concentration of the detergent below its critical micelle concentration. Each reconstitution method has its disadvantages. Dialysis can take weeks in cases where the detergent has low critical micelle concentration—this length of time can result in denaturation of the membrane proteins. Dilution results in large volumes, which subsequently lowers protein concentrations—making downstream experiments difficult. Detergent adsorption by polystyrene beads removes detergents efficiently, but due to its rapid and uncontrolled removal, can denature proteins. However, new promising methods have been proposed, which aim to remove these limitations, improve yield, and reduce reconstitution time.[56] Liposomes also can coencapsulate and co-deliver multiple-sized drug agents.[57] Coating liposomes with inert hydrophilic polymers (stealth liposomes) such poly(ethylene glycol) reduces nonspecific absorption and hence increases circulation times.[58] Although liposomes are promising stabilizing platforms for membrane proteins and drug delivery, their size (typically 100−500 nm for large unilamellar vesicles) can interfere with biosensor surfaces and hence decrease sensitivity in applications that require close proximity between the surface and sensor element (e.g., in SPR). However, this size limitation has been overcome by the development of planar lipid membranes, which can be constructed at sub-100 nm size ranges.[55] Supported-lipid bilayers, hybrid bilayer lipid membranes, polymer-cushion membranes, and tethered membranes are all different planar lipid models which aim to mimic the cell membrane structurally, allowing membrane proteins to retain their structural integrity and functionality across many protein applications.[59] These particular models require the use of solid supports in which different lipid compositions aim to provide a native-like environment for protein studies.

2.1.5. CHESS. Cellular high-throughput encapsulation, solubilization, and screening (CHESS) is a recently developed method that generates detergent-resistant containers from single cells, by physically enclosing detergent-solubilized integral membrane proteins (IMPs), which allows for direct selection of detergent-stable IMPs from diverse display libraries (Figure 3).[23,60] CHESS encapsulates single *Escherichia coli* (*E. coli*) cells from a library, with each expressing a different G protein-coupled receptor (GPCR) variant, to form detergent-resistant, semipermeable nanocontainers. These containers resist solubilization by detergents, which allow GPCRs to be solubilized in situ while maintaining an association with the protein's genetic information. The pore size of the nanocontainer is controlled to permit GPCR ligands to permeate, but also allowing the solubilized receptor to remain within the nanocapsule. Fluorescently labeled ligands are then used to bind to those GPCR variants inside the nanocontainers that remain active in detergent. Recently CHESS has been developed to evolve stable and soluble proteins directly.[60] The protein superfolder GFP was evolved using CHESS to become resistant to detergents including sodium dodecyl sulfate, high temperatures, and low pH. This allowed large libraries of soluble proteins to be directly screened for stability under these conditions. Some limitations remain, including the requirement to link a target protein to a fusion molecule so that its molecular weight is greater than 70 kDa (this prevents the protein from diffusing out of the nanocontainer). Although low-pH (<6) conditions resulted in some viable nanocapsules, many aggregated, which suggests that further optimizations remain in sample preparation and reducing exposure time to maximize the selection of viable soluble proteins in acidic conditions. High-pH (<9) conditions resulted in dissolutions of the nanocapsules, which suggests that alternative nanocapsule coatings would be required to select for viable proteins in high-pH conditions. Currently, CHESS also requires a fluorescent readout to determine protein stability for fluorescent activated cell sorting (FACS) selection. However, FACS is a powerful system for rapidly sorting cells, and as such, coupled with CHESS, allows for the direct selection of rare mutations that infer increases in protein stability under abnormal cell conditions. Together, these methods demonstrate the approach of using cell envelopes to stabilize membrane proteins in native or native-like lipid environments for protein studies.

3. MEMBRANE FRAGMENTS FROM MAMMALIAN CELLS

Mammalian membrane cells are other useful compositions that can be used in protein and cellular studies. There are several studies using membrane fragments from mammalian cells for fundamental studies such as cell−cell interaction, toxin absorption and cancer therapy to take advantage of the unique structure and property of cell plasma membranes.[61−66] Similar to stealth liposomes, cell membrane capsules (CMC) have been used to encapsulate chemotherapeutic drugs.[62,63] These drugs are typically rapidly cleared in their free form and lack cancer cell specificity. Synthetic nanoparticles have been commonly used to deliver these drugs, but can be recognized by the immune system. As such, natural approaches such as CMC allow for drug delivery while evading the body's defense mechanisms due to its structural composition of cell membrane proteins and lipids, allowing for good biocompatibility. CMCs can also be used for controlled loading release of encapsulated reagents.[63] CMCs are nontoxic and can effectively minimize recognition and internalization by macrophages, thus evading immune attack in the body. CMCs are intrinsically biocompatible and functional drug delivery and release vehicles. CMCs in the form of tumor cell-derived microparticles can also be used as vectors to deliver chemotherapeutic drugs. These cellular membrane microparticles are safer and self-friendlier, with reduced toxicity (i.e., do not induce autoimmunity). Their micrometer size is much larger than physiological capillary gaps that are around 5−8 nm, which prevents these micrometer-sized particles from reaching normal tissue and causing damage. Other applications of membrane compositions involved the use of mammalian cell-derived native vesicles as novel bioanalytical reagents that allow the miniaturization of receptor-based assays under physiological conditions.[65] Cultured mammalian cells are suitable for recombinant expression because they provide post-translational modifications essential for receptor function. These vesicle cell-surface receptors and cytoplasmic proteins retain their original cellular location, orientation, and function. They can be stored for weeks without losing their functional integrity. These vesicles could be used as a universal and inexpensive bioanalytical reagent for investigating cellular signaling reactions. Membrane fragment compositions comprised of polymeric core nanoparticles coated with bilayers of red blood cell (RBC) membranes have been used as "nanosponges" that absorbs cellular damaging toxins.[61] These RBC nanoparticles can absorb and neutralize a range of pore-forming protein toxins potentially resulting in improved therapeutic outcomes.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

Together these mammalian membrane fragment technologies demonstrate how mimicking native environments can be beneficial for drug delivery, cellular studies, and proteomic work. Mammalian membranes have many benefits including low toxicity for drug delivery and correct orientation of the fragment to the nanoparticle - which is important to maintain their biological performance. Other molecules (e.g., recombinant antibodies) which are native to other parts of a cell (e.g., yeast cell wall) can also benefit by encapsulation in a native or native-like environment during protein studies.

4. YEAST DISPLAY LIBRARY FOR SCFV SELECTION

Numerous recombinant technologies have been developed for selecting affinity binders from either large libraries of polypeptides or antibody fragments. scFvs and other affinity binders are readily isolated from these libraries, displayed using various systems, and then selected and expressed for further characterization. These capabilities enable isolation of antibody fragments chosen toward a particular target antigen without the need for animal immunization.[67−70]

Yeast cell display, introduced in 1997,[71] was developed to enhance affinity, stability properties such as affinity (i.e., affinity maturation), stability, and expression of proteins and is compatible with flow cytometry.[72] Yeast clones of interest are isolated based on the phenotype of binding fluorescently labeled antigen. The antigen-binding yeast can then be rapidly sorted using FACS. As such antigen-binding yeast clones can be selected from naïve libraries in as little as 2−3 weeks, eliminating the need for subcloning, expression, and purification steps.[72] This contrasts with phage display,[73,74] wherein the phage library is panned over an immobilized ligand and then washed and eluted in bulk. The ability to visualize binding in real time is highly advantageous because it enables ongoing monitoring and fine-tuning of selection strategies. Successful binding in phage[73,74] and ribosomal[75] display panning procedures cannot be assessed until the final wash step is complete and the binding fraction is eluted and propagated. In contrast, yeast selections are more dynamic because the library can be incubated with multiple antigen concentrations and under varying conditions, and the level of antigen binding can be assessed in real time by flow cytometry. This was illustrated by a study published in 2003, where yeast display was used for the discovery and characterization of novel affinity reagents from a large nonimmune, human scFv library.[76] The previously described advantages of FACS for rapid selection and flow cytometry for real-time assessment of binding, as well as the ability to develop whole yeast flow cytometric detection assays,[24] demonstrates that yeast display technology is a powerful technique for creating a library of antibody fragments.

4.1. Whole Yeast Cells Stabilizing Membrane Proteins for scFv Selection. Yeast display has also been used to screen antibody libraries against membrane proteins in their near-native conformations. Yeast biopanning, wherein monolayers of whole cells act as the antigen, has been suggested as a potential approach.[77] Typically yeast display requires the use of soluble antigen against which a library can be biopanned. As previously discussed, this poses a problem when attempting to select antibody fragments against membrane proteins. Combining yeast surface display directly with whole cells or detergent-solubilized whole-cell lysates can potentially solve this problem by allowing antibody libraries to be screened against membrane proteins in the near-native conformations.[78] One example is yeast biopanning; yeast-displayed scFvs were selected by successive rounds of incubation in mammalian monolayers, with nonspecifically bound yeast removed by washing. This yeast biopanning method was later used to isolate a number of unique scFv that bind to plasma membrane proteins of a rat brain endothelial cell line.[79]

Another approach employed yeast surface display-based screening using cell lysates as a soluble antigen source.[80] Detergent-solubilized membrane proteins in the form of cell lysate were mixed with scFv-displaying yeast, and the resulting mixture was sorted using FACS. This process was repeated leading to the enrichment of yeast clones that bound a desired antigen. Antibody fragments were then eluted from the yeast surface by yeast display immunoprecipitation (YDIP).[78] These methods demonstrate the efficient discovery of novel antibody-target combinations toward membrane proteins stabilized by whole-cells or solubilized whole-cell lysates.

4.2. Strategies for scFv Stabilization. A limitation of scFvs derived from yeast display libraries is the performance of recombinant antibody-like fragments in solution.[12,81] In yeast and phage display, antibody fragments are selected for affinity and stability when bound to surfaces. As a result, fragments which possess high activity on surfaces often lose their activity in solution. Although there are numerous examples of scFvs being used successfully in immunoassays,[82−88] many scFvs perform unsatisfactorily compared to full-length mAbs due to stability issues. Moreover, antibody fragments typically display low stability and are prone to aggregation due to a lack of interdomain stabilization which is found in larger antibody reagents such as IgG and Fab fragments.[7,15,89] Strategies for scFv stabilization vary. Some methods are detailed here and in Table 2.

4.2.1. Aggregation Resistance by Modifying Antigen Binding Sites. In a recent study, aggregation-resistant scFvs were produced by introducing negatively charged amino acids in the antigen binding sites.[16] Mutation additions of aspartate- or glutamate-enhanced aggregation resistance by altering the local charge distribution at highly specific positions, irrespective of sequence diversity at other positions. The mutant V_H and V_L domains were determined to be highly conserved, showing minimal impact on binding superantigen compared to non-mutant (wildtype) scFv before heating to 80 °C, and considerably improved superantigen binding compared to wildtype scFv after heating had been performed. Wildtype scFvs readily aggregated at high temperatures; however, this was not observed for mutant scFvs. This mutation allowed for improved expression and purification yields for the production of therapeutic antibody fragments; however, additional work is required to evaluate if these mutations improve the solubility of full-length antibodies. Similar work on full-length antibodies has shown that mutations introduced at specific complementarity determining regions (CDRs) on full-length antibodies can also increase antibody solubility and decrease aggregation.[90−93] Interestingly, introducing glycans into these antibodies (CDR and C_H1 domains) was found to increase solubility without reducing binding affinity.[90,92] Furthermore, glycan type was found to affect solubility, with IgGs expressed in yeast with mannose-rich glycans found to be more resistant to aggregation than the same IgGs expressed in mammalian cells.[94] One key study demonstrated that avoiding aggregation hotspots within antibodies requires not only consideration of static antibody structures but considerations of dynamics as well.[93] These investigators used molecular simulations of IgGs to identify aggregation-prone hydrophobic regions that were either natively

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

Table 2. Comparison of scFv Stabilization Strategies

scFv stabilization strategies	advantages	disadvantages
(a) aggregation resistance by modifying antigen binding sites	scFv stability improved by creating aggregation-resistant mutants	modifying antigen binding sites is cumbersome and could potentially impact antigen binding ability
(b) modifying net antibody charge	CDRs left unmodified; as such the impact on antigen binding ability is less likely to be affected	requires mutation of sites along an antibody—which is a still a cumbersome method
(c) cell envelope compositions	simple strategy to stabilize biomolecules (i.e., scFv), with limited to no further processing steps required—antibody fragment is left in its native cell envelope environment; can be kept intact or fragmented depending on application; could be lyophilized for long-term storage; cell surfaces allow for multiple scFvs, which provide a multivalent avidity effect for protein capture	not suited to some applications where the native cell wall could interfere with the surrounding environment; potential for cell fragments to aggregate; possibility of nonspecific absorption if fragment size is not optimized; considerations of biomolecule (i.e., scFv) cell-surface heterogeneity expression needs to be undertaken

exposed or exposed due to dynamic fluctuations or conformational changes. The simulations modeled the solvent exposure of every atom in an antibody and the relative aggregation propensity of each amino acid was calculated. These simulations identified aggregation hotspots in both the CDRs and constant domains. Further development of simulation models could improve identification of solubilizing mutations that do not impact binding affinity.

4.2.2. Modifying Net Antibody Charge. Another method to increase antibody solubility without mutating their CDRs is by increasing the net charge of an antibody.[17,95,96] In one study, scFvs were "supercharged" by mutating solvent-exposed residues to charged residues of the same polarity.[17] Using a computational program, researchers selected charge mutations in the V_H and V_L domains of a scFv that were predicted to avoid destabilizing the antibody fold. Interestingly, it was found that positively charged scFvs remained much more stable and retained high binding affinities when heated, compared to negatively charged scFvs or wild-type scFvs This observation does not apply to single-domain antibodies (dAbs), as aggregation-resistant dAbs typically have net negative charges due to their acidic isoelectric points.[16,95−98] These studies underscore the need to better understand how charged residues impact aggregation and solubility of antibodies and antibody fragments.

4.2.3. Cell Envelope Compositions. Stability can be achieved by keeping antibody fragments anchored to the display host (e.g., to the yeast cell wall).[24,25] This strategy is an alternative to the standard approach of solubilizing scFvs prior to anchoring them onto a surface.[99] Importantly, this approach maintains scFvs in the environment in which they were selected to function. Thus, in the case of using a yeast display library, the advantage of using FACS for selection is not undermined by reengineering the scFv into a soluble form. Lyophilized whole-cell yeast−scFv reagents, used in sandwich assay formats with traditional immunoglobulin signal antibodies, have been described.[24] More recently, whole yeast cells expressing scFv on their surface were modified with gold binding peptide, to allow a simple and cost-effective method for conjugation of the whole yeast cell sensor to a gold substrate.[30]

Whole-cell yeast−scFv probes that were lyophilized to create stable reagents that did not require refrigeration were immediately usable following rehydration.[24] With the development of improved lyophilization and storage protocols, whole-cell yeast−scFv were found to have a shelf life in lyophilized form at 45 °C for up to a year.[100] Furthermore, flow cytometry and immunofluorescence microscopy direct assays were developed that used whole-cell yeast−scFv reagents in combination with these common laboratory devices. However, these whole-cell reagents are insoluble and too large for many immunodiagnostic applications. Moreover, they require the use of labeled polyclonal or monoclonal antibodies to detect antigen binding to the yeast−scFv particles. Although it was not necessary that the detection antibodies be highly specific to the antigen (the yeast-scFv reagent conferred monoclonal specificity), the requirement for a traditional animal-derived detection antibody diluted the benefits of using yeast−scFv.

4.3. Nanosized Yeast Cell Envelope Antibody Compositions. The limitations of whole-cell yeast-scFv were addressed by mechanically fragmenting whole-cell yeast-scFv into nanosized yeast cell wall pieces to produce cell-free yeast-scFv affinity reagents.[25,26,28] Cell wall fragments bearing displayed scFv (Figure 4) become enriched by binding to surface-attached

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

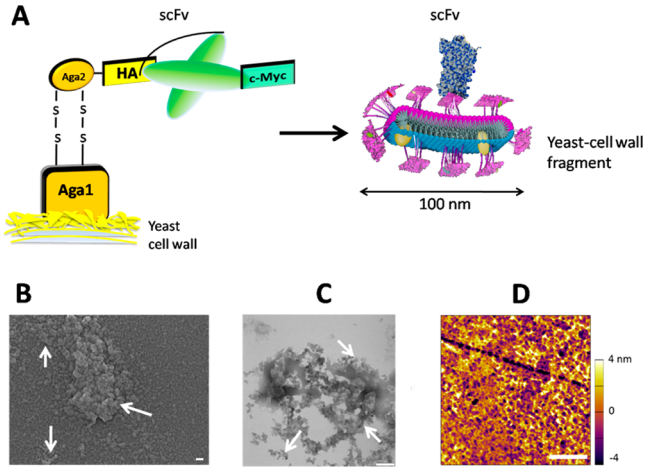


Figure 4. Schematic of nanoyeast–scFv particles. (A) Nanoyeast–scFv uses the Boder and Witrrup **a**-agglutinin display system to express scFv on the cell surface. As with whole yeast scFv, antibody fragments remain covalently attached to the fragments of yeast cell wall, but are filtered to <100 nm in size using a syringe pump size filter. (B) SEM image showing clusters of nanoyeast–scFv specifically immobilized onto a substrate. Scale bar = 100 nm. (C) TEM image showing clusters of nanoyeast–scFv eluted into solution. Nanoyeast–scFv are globular particles each less than 100 nm in size, but aggregating together into larger structures in solution. Scale bar = 200 nm. (D) AFM micrograph showing a nanoyeast–scFv cluster immobilized onto a surface. Scale bar = 500 nm. Arrows indicate the presence of globular nanoyeast–scFv in SEM and TEM images. Adapted with permission from ref 28. Copyright 2015 American Chemical Society.

antibodies specific to the scFv's epitope tags. These reagents, termed nanoyeast–scFv, have been developed as a new format for preparation of scFvs, have the potential as substitutes for full-length mAbs, and have demonstrated specific advantages.[25] As with whole-cell yeast–scFv fragments, nanoyeast–scFv retain the stability and functionality for which they were selected. The yeast surface display is used to express scFv on the cell surface by use of the **a**-agglutinin system that was developed by Boder and Wittrup[71,101] The Boder and Wittrup system uses Aga2 as the display fusion partner. Aga2 has a disulfide link to a glycosylphosphatidylinositol (GPI)/Iβ-1,6-glucan-anchored protein, named Aga1, which is covalently attached to the yeast cell wall (Figure 4A). This system secures the fusion protein to the yeast cell wall. Engineered into the scFv are N-terminal hemagglutinin (HA) and C-terminal c-Myc epitope tags, which enable detection and affinity purification of this fusion protein independently of its ligand-binding characteristics. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) were used to characterize the structure of nanoyeast–scFv (Figure 4B–D). Nanoyeast–scFv were specifically captured and immobilized by their HA tag using a HA antibody conjugated to a highly polished anatomically flat glass substrate. SEM shows clusters of nanoyeast–scFv as globular particles coated on the substrate surface (Figure 4B). For an "in-solution" characterization, nanoyeast–scFv were captured onto magnetic particles via their HA tag and then gently eluted off using excess HA peptide and analyzed using TEM (Figure 4B). Micrometer-sized aggregation of nanoyeast–scFv clusters was evident. Suggesting that more work to prevent aggregation in solution needs to be considered. AFM of nanoyeast–scFv immobilized onto an atomically flat polished glass substrate also showed globular particles that cover the sensor surface.

These novel reagents can be readily selected from yeast scFv antibody libraries (Figure 5). The cell wall fragment anchorages may constrain scFv secondary and tertiary structure,

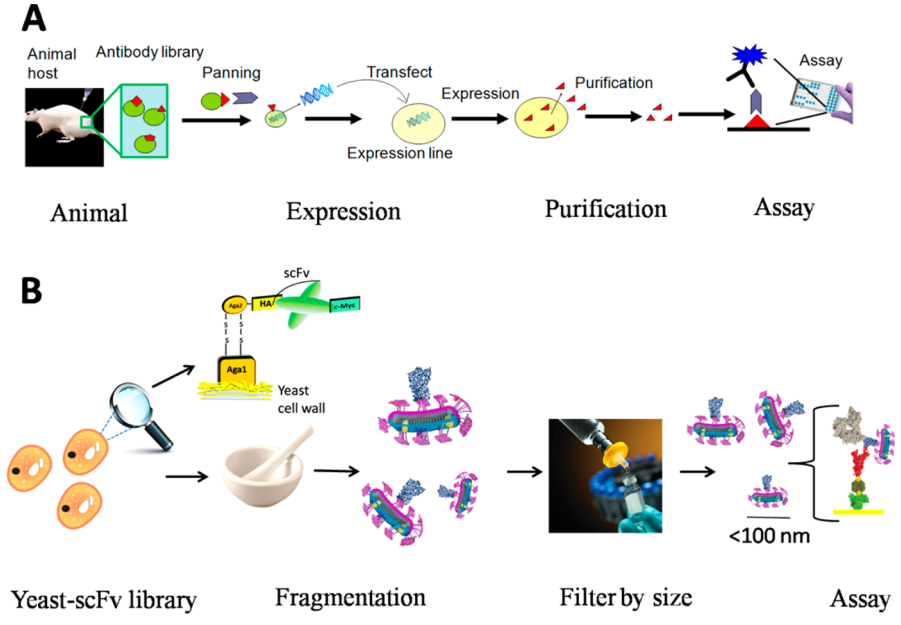


Figure 5. (A) Traditional antibody production method. The generalized outline shows the first step in inoculating an animal to form an immune response, all the way to the final stage of using the antibody in an assay. This entire process typically takes 2–3 months. (B) Production of nanoyeast–scFv is a simple process once scFvs are displayed on a yeast library. Yeast–scFv can be kept lyophilized a room temperature for up to a year. Once needed they can be simply fragmented using a mortar and pestle, resuspended, and filtered by size using a syringe filter. Nanoyeast–scFv can then be used directly in an immunoassay. This process from the yeast display library to nanoyeast–scFv production takes 2–3 weeks and just minutes from FACS selected yeast–scFv to nanoyeast–scFv. This recombinant production eliminates some of the steps required for generating antibodies.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649–30664

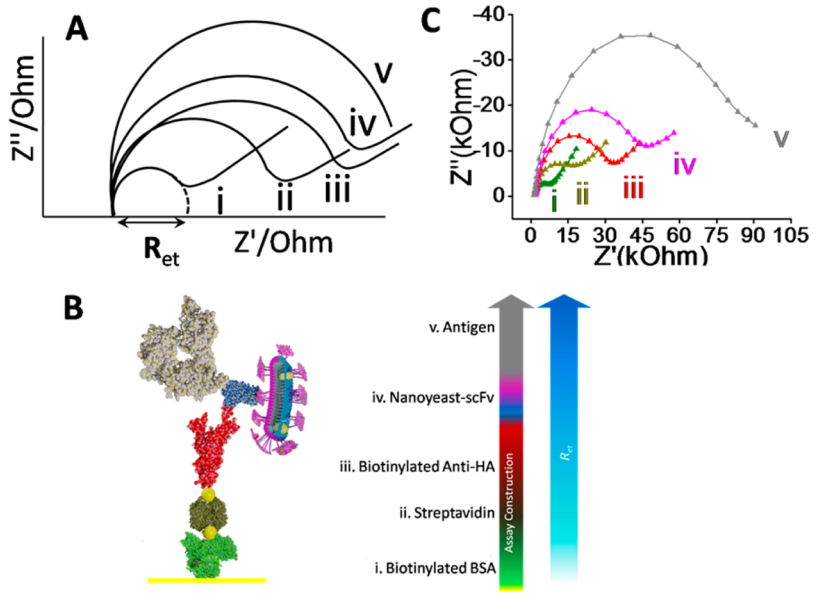


Figure 6. (A) Example of a Nyquist plot, which plots the real (Z') and imaginary (Z'') components of impedance measurements. As biomolecules at each step of an immunoassay are immobilized onto the biosensor layer on a conducting or semiconducting electrode, an increase in R_{et} is observed at each immobilization step. Here, we have an example of five different steps of an immunoassay being immobilized onto an electrode, which corresponds to a R_{et} increase at each step of i–v. (B) Construction of a nanoyeast–scFv biosensor, layer by layer, which causes the impedance to increase at each layer respectively as shown in the Nyquist plot in panel C. Sensor constructed on gold substrate. Panel B reprinted with permission from ref 26. Copyright 2014 Elsevier. Panel C adapted with permission from ref 25. Copyright 2013 The Royal Society of Chemistry.

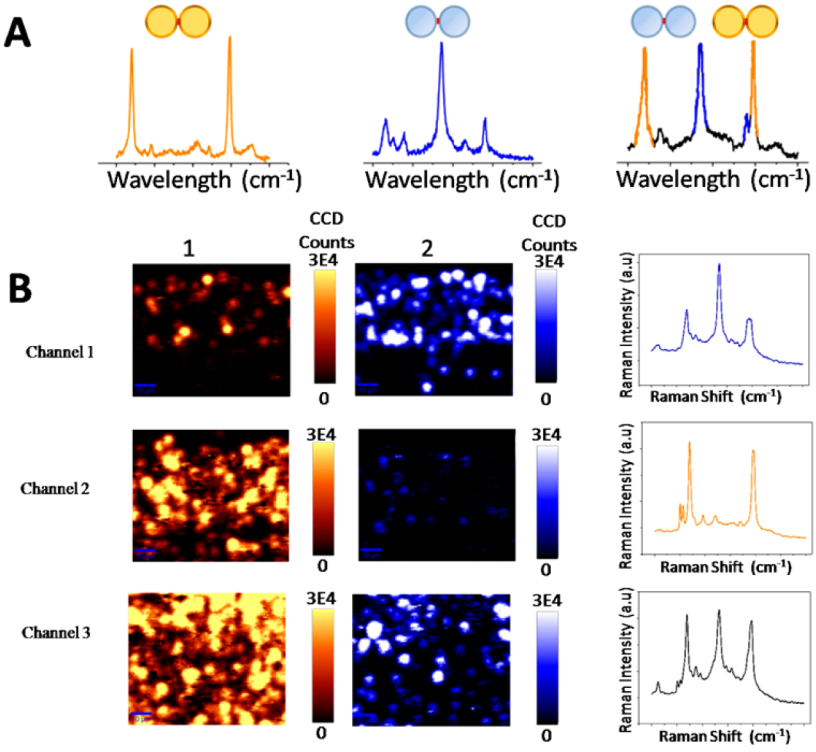


Figure 7. Example of SERS spectra. (A) SERS particles have unique spectra, which allow for multiplex detection. Here two sets of SERS reporter labels are shown with their unique spectra and then together, showing multiplex detection. (B) Nanoyeast–scFv affinity reagents were used for duplex detection of two different types of pathogen antigens in a three-channel microfluidic device. False-color images of SERS reporter particles binding to nanoyeast immobilized antigens, and their corresponding average SERS spectra are shown. Channels 1 and 2 each were coated with nanoyeast–scFv that were each specific toward one type of antigen. Channel 3 contained both types of nanoyeast–scFv, allowing for simultaneous duplex detection. Adapted with permission from ref 27. Copyright 2014 American Chemical Society.

holding the scFv in configurations that are best for antigen binding.[28] Cell fragments were determined to be globular from electron microscopy and atomic force microscopy (AFM) measurements (Figure 4B–D).[28] Similarly to the yeast display and mammalian cell technology highlighted earlier, the orientation of the protein (scFv) can be manipulated to the improve

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649–30664

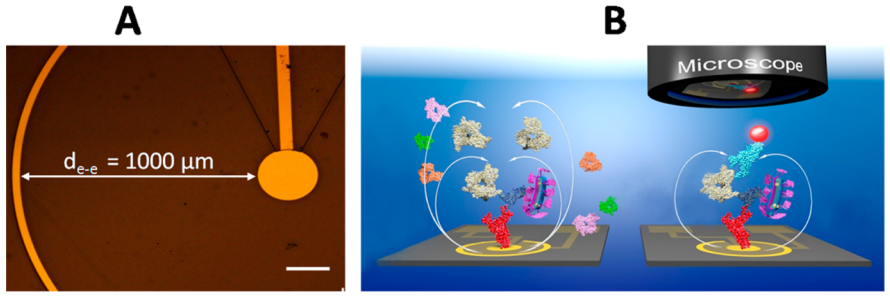


Figure 8. Schematic representation of the mechanism of ac-EHD induced surface shear forces for rapid capture and detection of antigen using nanoyeast−scFv as protein capture agents. (A) Optical image of the asymmetric electrode pair containing an inner circular small electrode and a large outer ring electrode with an edge to edge distance of 1000 μm between the electrodes. The diameters of the inner electrode and the width of the outer ring electrode were 250 and 30 μm, respectively. Scale bar = 200 μm. (B) Schematic representation of the mechanism of ac-EHD induced surface shear forces for rapid capture and detection of a pathogenic antigen (not drawn to scale). A confocal microscope visualized detection antibody conjugated with quantum dots. Figure reproduced with permission from ref 29. Copyright 2015 American Chemical Society.

display of functional properties. This can be achieved by linking the scFv by either the N- or C-terminus to the anchor protein.[102]

Nanoyeast−scFvs are generated by mechanical fragmentation using a mortar and pestle and then filtered by size to produce yeast cell wall fragments of varying sizes, with the scFv complex remaining intact. Cell fragment concentration can be determined using protein concentration assays, which are based on measuring proteins present in the yeast cell fragments. Cell fragment size can be controlled using size exclusion filters with a simple syringe pump. This method eliminates larger fragments (>220 nm) which have been found to interfere with electrochemical-based biosensors.[28] Larger size fragments may exert more force in solution, potentially overcoming the anti-HA bond that captures nanoyeast−scFv onto the biosensor, resulting in these larger cell wall fragments becoming dislodged from the biosensor surface.[28]

4.4. Yeast Cell Envelope Antibody Compositions in Biosensing Applications. An electrochemical approach has been utilized to detect antigen binding using nanoyeast−scFv (Figure 6) with a sensitivity of approximately 10 pg mL^{-1} using a $[Fe(CN)_6]^{3-/4-}$ redox probe.[26] Faradaic electrochemical impedance spectroscopy (F-EIS) allows the detection of capacitance changes for the label-free detection of biomolecules and probing the buildup of layers of the biomaterials on an electrode. Successful detection and capture of a biomolecule of interest can be observed as a change in the capacitance and interfacial electron-transfer resistance of an electrode. In a Randles equivalent circuit, impedance measurements are presented in the form of a Nyquist plot, with the real, Z', and imaginary components, Z'', and includes a semicircle region laying on the Z' axis followed by a straight line (Figure 6). At higher frequencies, the semicircle portion of the Nyquist plot is observed, which corresponds to the electron-transfer-limited process. The semicircle diameter is directly related to the electron-transfer resistance at the electrode surface, R_{et}.

Therefore, construction of an immunosensing layer and antibody/antigen complex binding can be observed by F-EIS, where the change in impedance of the electrode surface and electrolyte solution, containing a redox probe (e.g., $Fe(CN)_6]^{3-/4-}$) is measured in the form of its R_{et} (Figure 6).

The utility of nanoyeast−scFv as an antigen capture agent was further demonstrated by specifically capturing pathogen antigens which were spiked into a biological matrix comprised of stool.[26] In addition to the single pathogen antigen successfully captured in the previous work,[25] a new second pathogen antigen type was tested, and its respective cognate nanoyeast−scFv was developed.[26] This is consistent with the prediction that nanoyeast−scFv could be routinely engineered to capture any target antigen of interest. In addition, screen-printed gold electrodes were used as the diagnostic platform, which replaced the gold macroelectrodes from the previous work. This supports the expectation that nanoyeast−scFv can be utilized in a point-of-care diagnostic.[26] The flexibility of nanoyeast−scFv has been demonstrated by the use of alternative readout platforms. Surface-enhanced Raman scattering (SERS) was employed in duplex antigen detection using nanoyeast−scFv probes (Figure 7).[27]

A SERS nanoparticle label comprises a noble metal nanoparticle coated with Raman reporter molecules for identification based on their characteristic vibrational Raman spectrum. Compared with conventional immunoassays based on electrochemistry, fluorescence, and ELISA, SERS has a number of advantages. The first is multiplexing capability, due to a single laser excitation resulting in a narrow-band Raman spectral signature and a wide excitation wavelength. Second, SERS amplifies Raman signals up to 10−14 orders of magnitude, providing very high sensitivity. SERS also provides unique spectral fingerprint signatures of analytes, allowing for high specificity. scFv were immobilized onto a microfluidic chip into different channels depending on the scFv target type (Figure 7). Captured antigens were detected using a secondary detection antibody which was labeled with SERS particles. The SERS labels each provide unique spectra which can be used to distinguish particles from one another using a Raman microspectrometer. Due to the sensitivity advantages gained by SERS reporters, the limit for nanoyeast−scFv detection (LOD) was reduced to 1 pg mL^{-1}.

An alternating current electrohydrodynamics (ac-EHD) platform demonstrated rapid capture and sensitive detection of target antigen all together in less than 5 min.[29] Under an ac-EHD field, the charges induced within the electrical double layer of an electrode experience an electrical body force that drives the bulk fluid onto the inner circular electrode. This fluid flow transports target molecules or detection antibody in the bulk fluid and can continuously supply target molecules (i.e., increase sensor−target affinity interactions) onto the capture domain. Further, the fluid flow can be tuned using the applied ac field to achieve optimal fluid flow that can maximize device performance. This ability of ac-EHD flow can enable rapid capture and detection of target antigens. scFv were immobilized onto ac-EHD device via an affinity tag cloned into the scFv.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

An ac-EHD field was applied across the device, resulting in a flow field which caused target collisions between the immobilized scFv and the antigens in the stool sample and the removal of nonspecifically captured proteins and molecules. A secondary quantum-dot-labeled detection antibody was used to detect capture of target antigens. The device was imaged under a confocal microscope to obtain fluorescence images of detected antigen (Figure 8). This was the first demonstration of nanoyeast−scFv being combined with ac-EHD to facilitate rapid capture of antigen, removal of nonspecifically bound molecules, and detection of remaining bound antigen, all within 5 min. Furthermore, the sensitivity (limit of detection) of nanoyeast−scFv for antigen capture was improved to 100 fg mL^{-1}.

Structural characterization of nanoyeast−scFv has revealed that the optimal yeast fragment size for protein capture is between 50 and 100 nm in an electrochemical sensor; this limit is likely due to larger size yeast fragments exerting more force in solution and detaching from the biosensor.[28] It is possible that larger fragments may also result in more nonspecific absorption to the yeast cell wall. Yeast fragment size can be simply controlled by the use of a syringe-driven size filter unit.

While the cell wall fragment provides advantages in generating nanoyeast−scFv quicker and cheaper and with added stability, nanoyeast−scFv may be limited to in vitro diagnostic applications. The effect of nanoyeast−scFv in vivo has yet to be investigated; it is possible the yeast cell wall fragment could elicit an immune response when introduced into a host. For example, recombinant nonpathogenic *Saccharomyces cerevisiae* (*S. cerevisiae*) based vaccines have been used for a number of tumors and pathogens to drive innate immunity of the vaccine antigen.[103−107] β-Glucans found in the yeast cell wall act as an inherent adjuvant that activates dendritic cells that in turn elicit a robust immune response.[107] As such, nanoyeast−scFv (being comprised of yeast cell wall fragments) may also potentially be used as an adjuvant for vaccines.

Although these cell wall fragments are relatively easy to generate, their heterogeneity with respect to size and batch to batch variation of displayed scFv could pose a potential problem in producing these fragments reliably. The display of scFv on yeast surfaces is not homogeneous, resulting in sections of yeast cell wall that do not display any scFv for selection. Current methods overcome these issues by the use of an affinity purification step that captures nanoyeast−scFv fragments by virtue of the interaction between anti-HA antibody and the HA affinity tag cloned into the yeast-displayed scFv. This ensures only active nanoyeast−scFv are conjugated onto the immunosensor, allowing for specific capture of target antigen. As such, current experiments have indicated these fragments can be consistently fragmented leaving the associated scFv stable and active for protein capture.[25−27] Other affinity reagents are typically purified antibody, which trade added time and expense for a pure affinity reagent.

5. OTHER CELL ENVELOPE COMPOSITIONS IN BIOSENSOR APPLICATIONS

Whole-cell biosensors have been developed which display enzymes such as xylose dehydrogenase (XDH), glucose dehydrogenase, maltose, and organophosphorus hydrolase (OPH) on the surface of *E. coli*.[108−112] Anchoring enzymes to microbial cells such as *E. coli* eliminates the need for enzyme purification, while also providing stability to the display enzyme.[108,110] Enzymes displayed on *E. coli* surfaces can be achieved by the fusion to an anchoring motif—ice nucleation protein (INP).[108−112] This fusion to the *E. coli* cell wall via INP was found to increase XDH stability compared to cytoplasmic-free XHD,[108] and improve stability of OPH compared to free protein.[110] Electrochemical measurements were carried out in each *E. coli* biosensor platform, with high sensitivity, good specificity, and good enzyme stability reported.[108−112]

A similar approach can be used in *S. cerevisiae* yeast cells, with a-agglutinin used as a cell wall anchor motif.[113] A benefit to using yeast cells compared to *E. coli*, is eukaryotic yeast cell can display properly folded proteins due to the presence of chaperons. Glucose oxidase (GOx) was recombinantly expressed on the surface of whole yeast cells (GOx-yeast) and then conjugated to carbon nanotube modified glassy electrodes to create a whole-cell biosensor for glucose.[113] Cyclic voltammograms (CVs) were used to determine the limit of detection obtainable by GOx−yeast. The limit of detection was estimated for glucose spiked into buffer was 50 nM, which was determined to have high sensitivity compared to other GOx nanostructure modified electrodes.[113] Furthermore, GOx−yeast also demonstrated good stability, a wide pH range (pH 3.5−11.5), and good thermostability up to 56 °C over a short period. These stability findings could be attributed to the yeast cell wall, which stabilized the GOx under varying conditions.

Filamentous phage particles can be used directly as biosensor supports. Amino acids forming the N-terminal of a major coat protein, pVIII, of phage particles can be modified to display billions of random octapeptides, creating a "landscape library" of phage particles.[114] These octapeptides can bind proteins, enzymes, and cells.[115−117] As with traditional phage particles, these landscape phage particles can be selected by successive rounds of biopanning toward target antigens. These selected (purified) landscape phages could then be immobilized onto a substrate and used as protein capture agents to detect target antigens in a sandwich ELISA system.[115] By using whole landscape phage directly as protein capture probes, this method retains the protein binding octapeptides in their native phage wall environment. This reduces time and complexity in construction of a biosensor. Interestingly, removing the entire coating protein wall from the phage particle and immobilizing that cell wall composition on a substrate as a enzyme binder in a microarray are not as effective (sensitive) as keeping the whole landscape phage particle intact.[116] The drop in sensitivity could be attributed to the isolated cell coating wall having a lower surface area to bind enzymes as compared the intact spherical-shaped filamentous landscape phage. The density of polypeptides per area in the microarray landscape phage surface was found to be greater than traditional microarray immobilization peptide strategies.[116] Whole-cell landscape phage has also been used to specifically capture SW620 colorectal carcinoma cells onto an label-free EIS biosensor.[117] Each landscape phage contains 4000 copies of octapeptides—which translate to 4000 recognition sites. A multivalent effect can be achieved when targeting whole cells, as these cells contain many binding sites across their surface. This results in increased avidity, and affinity is possible due to the interaction of thousands of recognition sites on landscape phage to many binding sites on carcinoma cells.[117] These applications have shown phage particles can be mutated to bind biomolecules and cells of interest. However, given filamentous landscape phage particles can be up to 800−900 nm in length and only a few nanometers in diameter,[118] the string-like dimensions of these particles could pose a problem for some applications where micrometer-sized particles could interfere with biosensor sensitivity or specificity.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

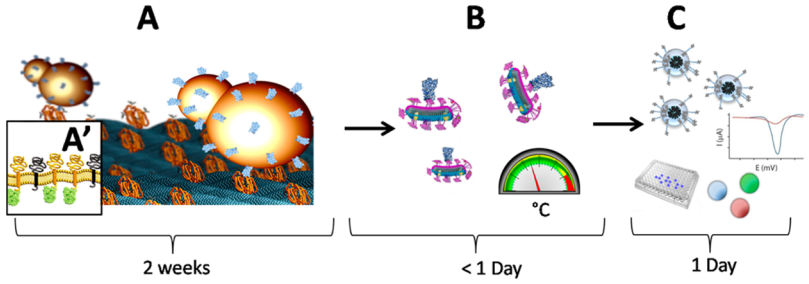


Figure 9. Integrated platform for the rapid deployment of cell envelope diagnostic reagents. (A) Yeast cells displaying scFvs are panned against cell membranes to select yeast−scFv binders onto cell membrane proteins. Transient transfection of alternating cell line CHO or HEK results in expression of the target membrane protein on the surface, with attached intercellular GFP (see inset A′), which increases the target protein density and provides a means to select for cells with high-level expression of cell-surface proteins using FACS. (B) Selected yeast cells are then fragmented into nanoyeast−scFv cell envelope compositions to produce soluble and temperature-stable reagents that can be used in (C) a range of assay platforms for the detection of disease.

Furthermore, previous work has shown decreased sensitivity in isolated cell coat protein octapeptides,[116] which suggests these particles are the most effective when intact and not in nanometer-sized fragments. Nonetheless, the applications demonstrated so far have shown that landscape phage has good scope as both a screening platform and a biosensing interface. As demonstrated in this review, cell envelope composition reagents can be reliably generated for use in biosensors.

6. LOOKING FORWARD—NOVEL PLATFORMS FOR RAPID DEVELOPMENT OF (FIELD-READY) DIAGNOSTIC REAGENTS

The global challenge now and in the future will be the rapid development of diagnostic tests for the detection of infectious disease. Better in-field diagnostics are required to monitor disease emergence and to track the progress of disease throughout populations. Furthermore, more than 50% of emerging diseases in humans over the past several decades are a result of transmittal between animals and humans;[119] Zoonotic disease emergence include more recent global health concerns regarding the spread of Ebola, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome coronavirus (MERS-CoV). Although there are concerted efforts by organizations such as The World Health Organization (WHO) to deploy diagnostics to monitor emerging infectious diseases in humans, and other organizations to monitor disease emergence in animals, there is little done to connect the two monitoring systems together to use animal sickness to predict human dieases.[120] As such, in addition to monitoring human population health, early and rapid field tests are required to enable health care task force officials and scientists to monitor potential animal-to-human pathogens.

Current approaches to vaccine, drug, and diagnostic development toward infectious diseases have been shown to be inadequate.[121−123] As of yet, no vaccine has ever been developed in time to change the course of an outbreak.[123] Government agencies are calling for flexible development and production platform technologies to improve R&D readiness for priority infectious disease threats.[121] As such, there is an absolute necessity for novel platform technologies whereby new antibodies can be rapidly isolated and incorporated into novel assay formats that are temperature, solution, and (biosensor) surface stable. Such reagents need to be seamlessly incorporated into cost-effective diagnostic devices that can be distributed and easily utilized in urban and rural environments.

In this review we have described state-of-the-art technologies which achieve these goals by creating nanostructures that comprise of antibody components and cell wall fragments. The nanoscaled cell wall fragment of these antibody composite structures renders exceptional temperature storage, and biosensor application stability. We now propose an integrated platform technology (Figure 9) that incorporates rapid isolation of antibodies using yeast display, followed by production and integration of nanoyeast−scFv into a device for rapid deployment. The ability to rapidly isolate antibodies using antibody display libraries technologies (bacteriophage, bacteria, yeast, and mammalian cell display) is well-established. However, the success of the technique is highly dependent on the quality of the antigen presented to the library, and isolation of antibodies against native membrane proteins can be challenging. We recently developed an antibody library biopanning technique that utilizes whole cells, which ensures recombinant membrane-bound proteins maintain their native conformation.[8] There are relatively few examples of successful cell-based biopanning methods in the literature, and most such methods fail to overcome the limitations of biopanning on the cell surface, namely, the low target density and the high background of irrelevant antigens.

This new methodology uses transient transfection of alternating host cell lines (CHO and HEK), as shown in Figure 9A, and stringent wash steps to allow for the selection of antibodies against membrane proteins in their native conformation. Once selections have been performed, scFvs can then be stabilized utilizing the previously described nanoyeast−scFv format. This new approach addresses the above limitations in the optimization of many cell-based panning methods. It allows for the rapid selection of temperature-stable cell diagnostic reagents against infectious disease targets in a simplified pipeline as compared to traditional mAb production.

Cell envelope compositions paired with rapid biopanning methods can address production time concerns by streamlining some of the required steps. The nanoyeast−scFv technology allows for the rapid development of mAbs toward infectious agents in a matter of 2−3 weeks from yeast library selection to incorporation into an assay (Figure 5B). The whole-cell biopanning using yeast−scFv libraries followed by a simple method for scFv stabilization through the production of nanoyeast−-scFv is a powerful platform technology for rapid isolation and production of affinity reagents, and is a step forward in addressing the limitations of drug and diagnostic reagent development.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

Developing a platform that rapidly selects antibodies against pathogen antigens presented on whole cells, and then stabilizing these temperature-stable reagents into cell envelopes for use in point-of-care diagnostics for monitoring of disease progression in animals and humans, can help potentially control disease emergence.

7. CONCLUSIONS

Cell envelope compositions have conferred stability on protein and antibody fragments by maintaining native or native-like environments during protein or immunoassay studies. Nanodiscs and other biological nanoparticles have demonstrated great utility in providing stability to membrane proteins by encapsulating these proteins in stabilizing phospholipids, allowing these membrane proteins to remain in solution and hence in a native-like environment. Cell-wall-based compositions have been developed in response to scFv solubilization challenges. Retaining a native yeast cell wall environment (i.e., a semirigid polysaccharide, lipid, and protein matrix) for yeast displayed scFvs has further stabilized these antibody fragments for use in diagnostic applications. Screening of yeast display libraries facilitates rapid isolation of high-affinity scFvs binders and then, crucially, correctly display the scFvs on the yeast membrane surface. In this way scFvs can be kept attached to the yeast cell wall, allowing these diagnostic agents to remain in the environment from which they were selected, thus providing much needed stability to these affinity binders. Nanoyeast−scFv can be lyophilized for long-term storage for up to a year. An assay can then be directly conducted on these lyophilized agents to reduce antibody production time. However, more advances will need to be made to this new production process to ensure that the reagents have the flexibility and sensitivity to stand alongside mAbs as alternative diagnostic protein capture agents. Currently, lyophilized, whole yeast−scFvs are insoluble and are too large for many diagnostic applications. Their readout method is also limited by the use of labeled secondary antibodies. To address these limitations, nanoyeast−scFvs were invented.[25−27] Nanoyeast−scFvs are designed as a new diagnostic protein capture agent used in immunosensors of disease biomarkers. These novel reagents build upon the work advanced by whole yeast−scFvs[24] but can overcome many of the limitations posed by that technology. Small, soluble agents are produced which can be used in a variety of molecular biosensing platforms, all while retaining the stability and quick time to manufacture and low-cost advantages presented by whole yeast−scFv. Together, these advances in cell envelope compositions have demonstrated that cell-surface proteins are best studied or utilized in their native environments. Furthermore, cell biopanning methods can be used to select antibodies toward membrane proteins in their native cell environment, allowing for the production of high-affinity binders. Cell envelope compositions could be developed toward other biomolecules that require native cell environments for stability.

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Notes

The authors declare the following competing financial interest(s): The nanoyeast-scFv work highlighted in this review is under patent as Cell-Free Biofragment Compositions and Related Systems, Devices, and Methods (WO 2014093357 A1).

ACKNOWLEDGMENTS

We acknowledge Sumukh Kumble for his assistance in designing the cell biopanning figure in this work. We gratefully thank all of our colleagues who have contributed over the years to the cited joint research work. This work was supported by the NIH, USA (Grant U01AIO82186-01), ARC DP (Grant DP140104006) and NHMRC CDF (Grant APP1088966). Although not directly funding the research work in this review, we acknowledge funding received by our laboratory from the National Breast Cancer Foundation of Australia (Grant CG-12-07). This grant has significantly contributed to the environment to stimulate the research described here.

REFERENCES

(1) Rask-Andersen, M.; Almén, M. S.; Schiöth, H. B. Trends in the Exploitation of Novel Drug Targets. *Nat. Rev. Drug Discovery* **2011**, *10* (8), 579−590.

(2) Bradbury, A.; Plückthun, A. Reproducibility: Standardize Antibodies Used in Research. *Nature* **2015**, *518* (7537), 27−29.

(3) Marx, V. Calling the next Generation of Affinity Reagents. *Nat. Methods* **2013**, *10* (9), 829−833.

(4) Weisser, N. E.; Hall, J. C. Applications of Single-Chain Variable Fragment Antibodies in Therapeutics and Diagnostics. *Biotechnol. Adv.* **2009**, *27* (4), 502−520.

(5) Bull, S. C.; Doig, A. J. Properties of Protein Drug Target Classes. *PLoS One* **2015**, *10* (3), e0117955.

(6) Hoogenboom, H. R. Selecting and Screening Recombinant Antibody Libraries. *Nat. Biotechnol.* **2005**, *23* (9), 1105−1116.

(7) Holliger, P.; Hudson, P. J. Engineered Antibody Fragments and the Rise of Single Domains. *Nat. Biotechnol.* **2005**, *23* (9), 1126−1136.

(8) Jones, M. L.; Alfaleh, M. A.; Kumble, S.; Zhang, S.; Osborne, G. W.; Yeh, M.; Arora, N.; Hou, J. J. C.; Howard, C. B.; Chin, D. Y.; Mahler, S. M. Targeting Membrane Proteins for Antibody Discovery Using Phage Display. *Sci. Rep.* **2016**, *6*, 26240.

(9) Ahmad, Z. A.; Yeap, S. K.; Ali, A. M.; Ho, W. Y.; Alitheen, N. B. M.; Hamid, M. scFv Antibody: Principles and Clinical Application. *Clin. Dev. Immunol.* **2012**, *2012*, 980250.

(10) Crivianu-Gaita, V.; Thompson, M. Aptamers, Antibody scFv, and Antibody Fab' Fragments: An Overview and Comparison of Three of the Most Versatile Biosensor Biorecognition Elements. *Biosens. Bioelectron.* **2016**, *85*, 32−45.

(11) Marx, V. Finding the Right Antibody for the Job. *Nat. Methods* **2013**, *10* (8), 703−707.

(12) Wörn, A.; Plückthun, A. Stability Engineering of Antibody Single-Chain Fv Fragments. *J. Mol. Biol.* **2001**, *305* (5), 989−1010.

(13) Willuda, J.; Honegger, A.; Waibel, R.; Schubiger, P. A.; Stahel, R.; Zangemeister-Wittke, U.; Plückthun, A. High Thermal Stability Is Essential for Tumor Targeting of Antibody Fragments: Engineering of a Humanized Anti-epithelial Glycoprotein-2 (Epithelial Cell Adhesion Molecule) Single-Chain Fv Fragment. *Cancer Res.* **1999**, *59* (22), 5758−5767.

(14) Wang, T.; Duan, Y. Probing the Stability-Limiting Regions of an Antibody Single-Chain Variable Fragment: A Molecular Dynamics Simulation Study. *Protein Eng., Des. Sel.* **2011**, *24* (9), 649−657.

(15) Lowe, D.; Dudgeon, K.; Rouet, R.; Schofield, P.; Jermutus, L.; Christ, D. Aggregation, Stability, and Formulation of Human Antibody Therapeutics. In *Advances in Protein Chemistry and Structural Biology*; Donev, R., Ed.; Academic Press: Oxford, U.K., 2011; Vol. *84*, pp 41−61.

(16) Dudgeon, K.; Rouet, R.; Kokmeijer, I.; Schofield, P.; Stolp, J.; Langley, D.; Stock, D.; Christ, D. General Strategy for the Generation

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

of Human Antibody Variable Domains with Increased Aggregation Resistance. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (27), 10879−10884.
(17) Miklos, A. E.; Kluwe, C.; Der, B. S.; Pai, S.; Sircar, A.; Hughes, R. A.; Berrondo, M.; Xu, J.; Codrea, V.; Buckley, P. E.; Calm, A. M.; Welsh, H. S.; Warner, C. R.; Zacharko, M. A.; Carney, J. P.; Gray, J. J.; Georgiou, G.; Kuhlman, B.; Ellington, A. D. Structure-Based Design of Supercharged, Highly Thermoresistant Antibodies. *Chem. Biol. (Oxford, U. K.)* **2012**, *19* (4), 449−455.
(18) Bayburt, T. H.; Sligar, S. G. Membrane Protein Assembly into Nanodiscs. *FEBS Lett.* **2010**, *584* (9), 1721−1727.
(19) Dominik, P. K.; Kossiakoff, A. A. Phage Display Selections for Affinity Reagents to Membrane Proteins in Nanodiscs. In *Methods in Enzymology*; Shukla, A. K., Ed.; Membrane Proteins—Engineering, Purification and Crystallization; Academic Press, 2015; Vol. *557*, Chapter 11, pp 219−245, DOI: 10.1016/bs.mie.2014.12.032.
(20) Vega, B.; Muñoz, L. M.; Holgado, B. L.; Lucas, P.; Rodríguez-Frade, J. M.; Calle, A.; Rodríguez-Fernández, J. L.; Lechuga, L. M.; Rodríguez, J. F.; Gutiérrez-Gallego, R.; Mellado, M. Technical Advance: Surface Plasmon Resonance-Based Analysis of CXCL12 Binding Using Immobilized Lentiviral Particles. *J. Leukocyte Biol.* **2011**, *90* (2), 399−408.
(21) Olguín, Y.; Carrascosa, L. G.; Lechuga, L. M.; Young, M. The Effects of Lipids and Surfactants on TLR5-Proteoliposome Functionality for Flagellin Detection Using Surface Plasmon Resonance Biosensing. *Talanta* **2014**, *126*, 136−144.
(22) Rigaud, J.-L.; Lévy, D. Reconstitution of Membrane Proteins into Liposomes. In *Methods Enzymol.*; Duzgunes, N., Ed.; Liposomes, Part B; Academic Press: San Diego, CA, USA, 2003; Vol. *372*, Chapter 4, pp 65−86, DOI: 10.1016/S0076-6879(03)72004-7.
(23) Scott, D. J.; Plückthun, A. Direct Molecular Evolution of Detergent-Stable G Protein-Coupled Receptors Using Polymer Encapsulated Cells. *J. Mol. Biol.* **2013**, *425* (3), 662−677.
(24) Gray, S. A.; Weigel, K. M.; Ali, I. K. M.; Lakey, A. A.; Capalungan, J.; Domingo, G. J.; Cangelosi, G. A. Toward Low-Cost Affinity Reagents: Lyophilized Yeast-scFv Probes Specific for Pathogen Antigens. *PLoS One* **2012**, *7* (2), e32042.
(25) Grewal, Y. S.; Shiddiky, M. J. A.; Gray, S. A.; Weigel, K. M.; Cangelosi, G. A.; Trau, M. Label-Free Electrochemical Detection of an Entamoeba Histolytica Antigen Using Cell-Free Yeast-scFv Probes. *Chem. Commun. (Cambridge, U. K.)* **2013**, *49* (15), 1551−1553.
(26) Grewal, Y. S.; Shiddiky, M. J. A.; Spadafora, L. J.; Cangelosi, G. A.; Trau, M. Nano-yeast−scFv Probes on Screen-Printed Gold Electrodes for Detection of Entamoeba Histolytica Antigens in a Biological Matrix. *Biosens. Bioelectron.* **2014**, *55*, 417−422.
(27) Wang, Y.; Rauf, S.; Grewal, Y. S.; Spadafora, L. J.; Shiddiky, M. J. A.; Cangelosi, G. A.; Schlücker, S.; Trau, M. Duplex Microfluidic SERS Detection of Pathogen Antigens with Nanoyeast Single-Chain Variable Fragments. *Anal. Chem. (Washington, DC, U. S.)* **2014**, *86* (19), 9930−9938.
(28) Grewal, Y. S.; Shiddiky, M. J. A.; Spadafora, L. J.; Cangelosi, G. A.; Trau, M. Structural Characterization of Nanoyeast Single-Chain Fragment Variable Affinity Reagents. *J. Phys. Chem. C* **2015**, *119* (22), 12674−12680.
(29) Vaidyanathan, R.; Rauf, S.; Grewal, Y. S.; Spadafora, L. J.; Shiddiky, M. J. A.; Cangelosi, G. A.; Trau, M. Enhancing Protein Capture Using a Combination of Nanoyeast Single-Chain Fragment Affinity Reagents and Alternating Current Electrohydrodynamic Forces. *Anal. Chem. (Washington, DC, U. S.)* **2015**, *87* (23), 11673−11681.
(30) Venkatesh, A. G.; Sun, A.; Brickner, H.; Looney, D.; Hall, D. A.; Aronoff-Spencer, E. Yeast Dual-Affinity Biobricks: Progress towards Renewable Whole-Cell Biosensors. *Biosens. Bioelectron.* **2015**, *70*, 462−468.
(31) Orlean, P. Architecture and Biosynthesis of the Saccharomyces Cerevisiae Cell Wall. *Genetics* **2012**, *192* (3), 775−818.
(32) Marty, M. T.; Wilcox, K. C.; Klein, W. L.; Sligar, S. G. Nanodisc-Solubilized Membrane Protein Library Reflects the Membrane Proteome. *Anal. Bioanal. Chem.* **2013**, *405* (12), 4009−4016.
(33) Yıldırım, M. A.; Goh, K.-I.; Cusick, M. E.; Barabási, A.-L.; Vidal, M. Drug—target Network. *Nat. Biotechnol.* **2007**, *25* (10), 1119−1126.
(34) Kinch, M. S. An Overview of FDA-Approved Biologics Medicines. *Drug Discovery Today* **2015**, *20* (4), 393−398.
(35) Reichert, J. M. Antibodies to Watch in 2016. *mAbs* **2016**, *8* (2), 197−204.
(36) Koenig, P.; Fuh, G. Selection and Screening Using Antibody Phage Display Libraries. *Methods Mol. Biol. (N. Y., NY, U. S.)* **2014**, *1131*, 133−149.
(37) Van Deventer, J. A.; Kelly, R. L.; Rajan, S.; Wittrup, K. D.; Sidhu, S. S. A Switchable Yeast Display/Secretion System. *Protein Eng., Des. Sel.* **2015**, *28* (10), 317−325.
(38) White, S. H.; Wimley, W. C. Membrane Protein Folding and Stability: Physical Principles. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 319−365.
(39) Tate, C. G. Practical Considerations of Membrane Protein Instability during Purification and Crystallisation. *Methods Mol. Biol. (N. Y., NY, U. S.)* **2010**, *601*, 187−203.
(40) Ritchie, T. K.; Grinkova, Y. V.; Bayburt, T. H.; Denisov, I. G.; Zolnerciks, J. K.; Atkins, W. M.; Sligar, S. G. Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs. In *Methods in Enzymology*; Düzgünes, N., Ed.; Liposomes, Part F; Academic Press: San Diego, CA, USA, 2009; Vol. *464*, Chapter 11, pp 211−231, DOI: 10.1016/S0076-6879(09)64011-8.
(41) Schuler, M. A.; Denisov, I. G.; Sligar, S. G. Nanodiscs as a New Tool to Examine Lipid-Protein Interactions. *Methods Mol. Biol. (N. Y., NY, U. S.)* **2013**, *974*, 415−433.
(42) Denisov, I. G.; Sligar, S. G. Nanodiscs for Structural and Functional Studies of Membrane Proteins. *Nat. Struct. Mol. Biol.* **2016**, *23* (6), 481−486.
(43) Borch, J.; Torta, F.; Sligar, S. G.; Roepstorff, P. Nanodiscs for Immobilization of Lipid Bilayers and Membrane Receptors: Kinetic Analysis of Cholera Toxin Binding to a Glycolipid Receptor. *Anal. Chem. (Washington, DC, U. S.)* **2008**, *80* (16), 6245−6252.
(44) Das, A.; Zhao, J.; Schatz, G. C.; Sligar, S. G.; Van Duyne, R. P. Screening of Type I and II Drug Binding to Human Cytochrome P450−3A4 in Nanodiscs by Localized Surface Plasmon Resonance Spectroscopy. *Anal. Chem. (Washington, DC, U. S.)* **2009**, *81* (10), 3754−3759.
(45) Reichart, T. M.; Baksh, M. M.; Rhee, J.-K.; Fiedler, J. D.; Sligar, S. G.; Finn, M. G.; Zwick, M. B.; Dawson, P. E. Trimerization of the HIV Transmembrane Domain in Lipid Bilayers Modulates Broadly Neutralizing Antibody Binding. *Angew. Chem., Int. Ed.* **2016**, *55* (8), 2688−2692.
(46) Denisov, I. G.; Grinkova, Y. V.; Lazarides, A. A.; Sligar, S. G. Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. *J. Am. Chem. Soc.* **2004**, *126* (11), 3477−3487.
(47) Knowles, T. J.; Finka, R.; Smith, C.; Lin, Y.-P.; Dafforn, T.; Overduin, M. Membrane Proteins Solubilized Intact in Lipid Containing Nanoparticles Bounded by Styrene Maleic Acid Copolymer. *J. Am. Chem. Soc.* **2009**, *131* (22), 7484−7485.
(48) Lee, S. C.; Knowles, T. J.; Postis, V. L. G.; Jamshad, M.; Parslow, R. A.; Lin, Y.; Goldman, A.; Sridhar, P.; Overduin, M.; Muench, S. P.; Dafforn, T. R. A Method for Detergent-Free Isolation of Membrane Proteins in Their Local Lipid Environment. *Nat. Protoc.* **2016**, *11* (7), 1149−1162.
(49) Scheidelaar, S.; Koorengevel, M. C.; Pardo, J. D.; Meeldijk, J. D.; Breukink, E.; Killian, J. A. Molecular Model for the Solubilization of Membranes into Nanodisks by Styrene Maleic Acid Copolymers. *Biophys. J.* **2015**, *108* (2), 279−290.
(50) Jamshad, M.; Grimard, V.; Idini, I.; Knowles, T. J.; Dowle, M. R.; Schofield, N.; Sridhar, P.; Lin, Y.; Finka, R.; Wheatley, M.; Thomas, O. R. T.; Palmer, R. E.; Overduin, M.; Govaerts, C.; Ruysschaert, J.-M.; Edler, K. J.; Dafforn, T. R. Structural Analysis of a Nanoparticle Containing a Lipid Bilayer Used for Detergent-Free Extraction of Membrane Proteins. *Nano Res.* **2015**, *8* (3), 774−789.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

(51) Vega, B.; Calle, A.; Sánchez, A.; Lechuga, L. M.; Ortiz, A. M.; Armelles, G.; Rodríguez-Frade, J. M.; Mellado, M. Real-Time Detection of the Chemokine CXCL12 in Urine Samples by Surface Plasmon Resonance. *Talanta* **2013**, *109*, 209−215.
(52) Martínez-Muñoz, L.; Barroso, R.; Dyrhaug, S. Y.; Navarro, G.; Lucas, P.; Soriano, S. F.; Vega, B.; Costas, C.; Muñoz-Fernández, M. Á.; Santiago, C.; Frade, J. M. R.; Franco, R.; Mellado, M. CCR5/CD4/CXCR4 Oligomerization Prevents HIV-1 gp120IIIB Binding to the Cell Surface. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (19), E1960−E1969.
(53) Olguín, Y.; Villalobos, P.; Carrascosa, L. G.; Young, M.; Valdez, E.; Lechuga, L.; Galindo, R. Detection of Flagellin by Interaction with Human Recombinant TLR5 Immobilized in Liposomes. *Anal. Bioanal. Chem.* **2013**, *405* (4), 1267−1281.
(54) Bally, M.; Bailey, K.; Sugihara, K.; Grieshaber, D.; Vörös, J.; Städler, B. Liposome and Lipid Bilayer Arrays Towards Biosensing Applications. *Small* **2010**, *6* (22), 2481−2497.
(55) Lee, Y. K.; Lee, H.; Nam, J.-M. Lipid-Nanostructure Hybrids and Their Applications in Nanobiotechnology. *NPG Asia Mater.* **2013**, *5* (5), e48.
(56) Althoff, T.; Davies, K. M.; Schulze, S.; Joos, F.; Kühlbrandt, W. GRecon: A Method for the Lipid Reconstitution of Membrane Proteins. *Angew. Chem., Int. Ed.* **2012**, *51* (33), 8343−8347.
(57) He, Q.; Guo, S.; Qian, Z.; Chen, X. Development of Individualized Anti-Metastasis Strategies by Engineering Nanomedicines. *Chem. Soc. Rev.* **2015**, *44* (17), 6258−6286.
(58) Zylberberg, C.; Matosevic, S. Pharmaceutical Liposomal Drug Delivery: A Review of New Delivery Systems and a Look at the Regulatory Landscape. *Drug Delivery* **2016**, *0* (0), 1−11.
(59) Rebaud, S.; Maniti, O.; Girard-Egrot, A. P. Tethered Bilayer Lipid Membranes (tBLMs): Interest and Applications for Biological Membrane Investigations. *Biochimie* **2014**, *107* (Part A), 135−142.
(60) Yong, K. J.; Scott, D. J. Rapid Directed Evolution of Stabilized Proteins with Cellular High-Throughput Encapsulation Solubilization and Screening (CHESS). *Biotechnol. Bioeng.* **2015**, *112* (3), 438−446.
(61) Hu, C.-M. J.; Fang, R. H.; Copp, J.; Luk, B. T.; Zhang, L. A Biomimetic Nanosponge That Absorbs Pore-Forming Toxins. *Nat. Nanotechnol.* **2013**, *8* (5), 336−340.
(62) Peng, L.-H.; Zhang, Y.-H.; Han, L.-J.; Zhang, C.-Z.; Wu, J.-H.; Wang, X.-R.; Gao, J.-Q.; Mao, Z.-W. Cell Membrane Capsules for Encapsulation of Chemotherapeutic and Cancer Cell Targeting in Vivo. *ACS Appl. Mater. Interfaces* **2015**, *7* (33), 18628−18637.
(63) Mao, Z.; Cartier, R.; Hohl, A.; Farinacci, M.; Dorhoi, A.; Nguyen, T.-L.; Mulvaney, P.; Ralston, J.; Kaufmann, S. H. E.; Möhwald, H.; Wang, D. Cells as Factories for Humanized Encapsulation. *Nano Lett.* **2011**, *11* (5), 2152−2156.
(64) Tang, K.; Zhang, Y.; Zhang, H.; Xu, P.; Liu, J.; Ma, J.; Lv, M.; Li, D.; Katirai, F.; Shen, G.-X.; Zhang, G.; Feng, Z.-H.; Ye, D.; Huang, B. Delivery of Chemotherapeutic Drugs in Tumour Cell-Derived Microparticles. *Nat. Commun.* **2012**, *3*, 1282.
(65) Pick, H.; Schmid, E. L.; Tairi, A.-P.; Ilegems, E.; Hovius, R.; Vogel, H. Investigating Cellular Signaling Reactions in Single Attoliter Vesicles. *J. Am. Chem. Soc.* **2005**, *127* (9), 2908−2912.
(66) Boilard, E.; Nigrovic, P. A.; Larabee, K.; Watts, G. F. M.; Coblyn, J. S.; Weinblatt, M. E.; Massarotti, E. M.; Remold-O'Donnell, E.; Farndale, R. W.; Ware, J.; Lee, D. M. Platelets Amplify Inflammation in Arthritis via Collagen-Dependent Microparticle Production. *Science (Washington, DC, U. S.)* **2010**, *327* (5965), 580−583.
(67) Marks, J. D.; Hoogenboom, H. R.; Bonnert, T. P.; McCafferty, J.; Griffiths, A. D.; Winter, G. By-Passing Immunization: Human Antibodies from V-Gene Libraries Displayed on Phage. *J. Mol. Biol.* **1991**, *222* (3), 581−597.
(68) Griffiths, A. D.; Williams, S. C.; Hartley, O.; Tomlinson, I. M.; Waterhouse, P.; Crosby, W. L.; Kontermann, R. E.; Jones, P. T.; Low, N. M.; Allison, T. J. Isolation of High Affinity Human Antibodies Directly from Large Synthetic Repertoires. *EMBO J.* **1994**, *13* (14), 3245−3260.
(69) Davies, J.; Riechmann, L. Antibody VH Domains as Small Recognition Units. *Bio/Technology* **1995**, *13* (5), 475−479.
(70) Reiter, Y.; Schuck, P.; Boyd, L. F.; Plaksin, D. An Antibody Single-Domain Phage Display Library of a Native Heavy Chain Variable Region: Isolation of Functional Single-Domain VH Molecules with a Unique Interface. *J. Mol. Biol.* **1999**, *290* (3), 685−698.
(71) Boder, E. T.; Wittrup, K. D. Yeast Surface Display for Screening Combinatorial Polypeptide Libraries. *Nat. Biotechnol.* **1997**, *15* (6), 553−557.
(72) Feldhaus, M. J.; Siegel, R. W. Yeast Display of Antibody Fragments: A Discovery and Characterization Platform. *J. Immunol. Methods* **2004**, *290* (1−2), 69−80.
(73) McCafferty, J.; Griffiths, A. D.; Winter, G.; Chiswell, D. J. Phage Antibodies: Filamentous Phage Displaying Antibody Variable Domains. *Nature* **1990**, *348* (6301), 552−554.
(74) Winter, G.; Griffiths, A. D.; Hawkins, R. E.; Hoogenboom, H. R. Making Antibodies by Phage Display Technology. *Annu. Rev. Immunol.* **1994**, *12* (1), 433−455.
(75) Mattheakis, L. C.; Bhatt, R. R.; Dower, W. J. An in Vitro Polysome Display System for Identifying Ligands from Very Large Peptide Libraries. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91* (19), 9022−9026.
(76) Feldhaus, M. J.; Siegel, R. W.; Opresko, L. K.; Coleman, J. R.; Feldhaus, J. M. W.; Yeung, Y. A.; Cochran, J. R.; Heinzelman, P.; Colby, D.; Swers, J.; Graff, C.; Wiley, H. S.; Wittrup, K. D. Flow-Cytometric Isolation of Human Antibodies from a Nonimmune Saccharomyces Cerevisiae Surface Display Library. *Nat. Biotechnol.* **2003**, *21* (2), 163−170.
(77) Wang, X. X.; Shusta, E. V. The Use of scFv-Displaying Yeast in Mammalian Cell Surface Selections. *J. Immunol. Methods* **2005**, *304* (1−2), 30−42.
(78) Tillotson, B. J.; Cho, Y. K.; Shusta, E. V. Cells and Cell Lysates: A Direct Approach for Engineering Antibodies against Membrane Proteins Using Yeast Surface Display. *Methods (Amsterdam, Neth.)* **2013**, *60* (1), 27−37.
(79) Wang, X. X.; Cho, Y. K.; Shusta, E. V. Mining a Yeast Library for Brain Endothelial Cell-Binding Antibodies. *Nat. Methods* **2007**, *4* (2), 143−145.
(80) Cho, Y. K.; Shusta, E. V. Antibody Library Screens Using Detergent-Solubilized Mammalian Cell Lysates as Antigen Sources. *Protein Eng., Des. Sel.* **2010**, *23* (7), 567−577.
(81) Bradbury, A. scFvs and beyond. *Drug Discovery Today* **2003**, *8* (16), 737−739.
(82) Joung, C.-K.; Kim, H.-N.; Im, H.-C.; Kim, H.-Y.; Oh, M.-H.; Kim, Y.-R. Ultra-Sensitive Detection of Pathogenic Microorganism Using Surface-Engineered Impedimetric Immunosensor. *Sens. Actuators, B* **2012**, *161* (1), 824−831.
(83) Shiddiky, M. J. A.; Kithva, P. H.; Kozak, D.; Trau, M. An Electrochemical Immunosensor to Minimize the Nonspecific Adsorption and to Improve Sensitivity of Protein Assays in Human Serum. *Biosens. Bioelectron.* **2012**, *38* (1), 132−137.
(84) Shang, Y.; Mernaugh, R.; Zeng, X. Characterization of the Native and Denatured Herceptin by Enzyme Linked Immunosorbent Assay and Quartz Crystal Microbalance Using a High-Affinity Single Chain Fragment Variable Recombinant Antibody. *Anal. Chem. (Washington, DC, U. S.)* **2012**, *84* (19), 8164−8170.
(85) Heo, N. S.; Zheng, S.; Yang, M.; Lee, S. J.; Lee, S. Y.; Kim, H.-J.; Park, J. Y.; Lee, C.-S.; Park, T. J. Label-Free Electrochemical Diagnosis of Viral Antigens with Genetically Engineered Fusion Protein. *Sensors* **2012**, *12* (8), 10097−10108.
(86) Al-Halabi, L.; Balck, A.; Michalzik, M.; Fröde, D.; Büttgenbach, S.; Hust, M.; Schirrmann, T.; Dübel, S. Recombinant Antibody Fragments Allow Repeated Measurements of C-Reactive Protein with a Quartz Crystal Microbalance Immunosensor. *mAbs* **2013**, *5* (1), 140−149.
(87) Spain, E.; Gilgunn, S.; Sharma, S.; Adamson, K.; Carthy, E.; O'Kennedy, R.; Forster, R. J. Detection of Prostate Specific Antigen Based on Electrocatalytic Platinum Nanoparticles Conjugated to a Recombinant scFv Antibody. *Biosens. Bioelectron.* **2016**, *77*, 759−766.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664

(88) Raftery, L. J.; Grewal, Y. S.; Howard, C. B.; Jones, M. L.; Shiddiky, M. J. A.; Carrascosa, L. G.; Thurecht, K. J.; Mahler, S. M.; Trau, M. Biosensing Made Easy with PEG-Targeted Bi-Specific Antibodies. *Chem. Commun. (Cambridge, U. K.)* **2016**, *52* (33), 5730−5733.
(89) Ewert, S.; Huber, T.; Honegger, A.; Plückthun, A. Biophysical Properties of Human Antibody Variable Domains. *J. Mol. Biol.* **2003**, *325* (3), 531−553.
(90) Wu, S.-J.; Luo, J.; O'Neil, K. T.; Kang, J.; Lacy, E. R.; Canziani, G.; Baker, A.; Huang, M.; Tang, Q. M.; Raju, T. S.; Jacobs, S. A.; Teplyakov, A.; Gilliland, G. L.; Feng, Y. Structure-Based Engineering of a Monoclonal Antibody for Improved Solubility. *Protein Eng., Des. Sel.* **2010**, *23* (8), 643−651.
(91) Bethea, D.; Wu, S.-J.; Luo, J.; Hyun, L.; Lacy, E. R.; Teplyakov, A.; Jacobs, S. A.; O'Neil, K. T.; Gilliland, G. L.; Feng, Y. Mechanisms of Self-Association of a Human Monoclonal Antibody CNTO607. *Protein Eng., Des. Sel.* **2012**, *25* (10), 531−537.
(92) Pepinsky, R. B.; Silvian, L.; Berkowitz, S. A.; Farrington, G.; Lugovskoy, A.; Walus, L.; Eldredge, J.; Capili, A.; Mi, S.; Graff, C.; Garber, E. Improving the Solubility of Anti-LINGO-1 Monoclonal Antibody Li33 by Isotype Switching and Targeted Mutagenesis. *Protein Sci.* **2010**, *19* (5), 954−966.
(93) Chennamsetty, N.; Voynov, V.; Kayser, V.; Helk, B.; Trout, B. L. Design of Therapeutic Proteins with Enhanced Stability. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (29), 11937−11942.
(94) Schaefer, J. V.; Plückthun, A. Engineering Aggregation Resistance in IgG by Two Independent Mechanisms: Lessons from Comparison of Pichia Pastoris and Mammalian Cell Expression. *J. Mol. Biol.* **2012**, *417* (4), 309−335.
(95) Perchiacca, J. M.; Bhattacharya, M.; Tessier, P. M. Mutational Analysis of Domain Antibodies Reveals Aggregation Hotspots within and near the Complementarity Determining Regions. *Proteins: Struct., Funct., Genet.* **2011**, *79* (9), 2637−2647.
(96) Perchiacca, J. M.; Ladiwala, A. R. A.; Bhattacharya, M.; Tessier, P. M. Aggregation-Resistant Domain Antibodies Engineered with Charged Mutations near the Edges of the Complementarity-Determining Regions. *Protein Eng., Des. Sel.* **2012**, *25* (10), 591−602.
(97) Jespers, L.; Schon, O.; Famm, K.; Winter, G. Aggregation-Resistant Domain Antibodies Selected on Phage by Heat Denaturation. *Nat. Biotechnol.* **2004**, *22* (9), 1161−1165.
(98) Dudgeon, K.; Famm, K.; Christ, D. Sequence Determinants of Protein Aggregation in Human VH Domains. *Protein Eng., Des. Sel.* **2009**, *22* (3), 217−220.
(99) Cho, Y. K.; Chen, I.; Wei, X.; Li, L.; Shusta, E. V. A Yeast Display Immunoprecipitation Method for Efficient Isolation and Characterization of Antigens. *J. Immunol. Methods* **2009**, *341* (1−2), 117−126.
(100) Kahn, M.; Priddy, S.; Estrada, M.; Spadafora, L.; Cangelosi, G. A.; Domingo, G. J. Methodology for Preservation of Yeast-Bound Single Chain Fragment Variable Antibody Affinity Reagents. *J. Immunol. Methods* **2015**, *427*, 134−137.
(101) Boder, E. T.; Wittrup, K. D. Yeast Surface Display for Directed Evolution of Protein Expression, Affinity, and Stability. In *Methods Enzymology*; Thorner, J., Emr, S. D., Abelson, J. N., Eds.; Applications of Chimeric Genes and Hybrid Proteins-Part C: Protein-Protein Interactions and Genomics; Academic Press: San Diego, CA, USA, 2000; Vol. *328*, Chapter 25, pp 430−444, DOI: 10.1016/S0076-6879(00)28410-3.
(102) Shusta, E. V.; Pepper, L. R.; Cho, Y. K.; Boder, E. T. A Decade of Yeast Surface Display Technology: Where Are We Now? *Comb. Chem. High Throughput Screening* **2008**, *11* (2), 127−134.
(103) Grover, A.; McLean, J. L.; Troudt, J. M.; Foster, C.; Izzo, L.; Creissen, E.; MacDonald, E.; Troy, A.; Izzo, A. A. Heat Killed Saccharomyces Cerevisiae as an Adjuvant for the Induction of Vaccine-Mediated Immunity against Infection with Mycobacterium Tuberculosis. *Vaccine* **2016**, *34* (25), 2798−2805.
(104) Liu, M.; Clemons, K. V.; Bigos, M.; Medovarska, I.; Brummer, E.; Stevens, D. A. Immune Responses Induced by Heat Killed Saccharomyces Cerevisiae: A Vaccine against Fungal Infection. *Vaccine* **2011**, *29* (9), 1745−1753.
(105) Capilla, J.; Clemons, K. V.; Liu, M.; Levine, H. B.; Stevens, D. A. Saccharomyces Cerevisiae as a Vaccine against Coccidioidomycosis. *Vaccine* **2009**, *27* (27), 3662−3668.
(106) Assis-Marques, M. A.; Oliveira, A. F.; Ruas, L. P.; Reis, T. F. d.; Roque-Barreira, M. C.; Coelho, P. S. R. Saccharomyces cerevisiae Expressing Gp43 Protects Mice against Paracoccidioides brasiliensis Infection. *PLoS One* **2015**, *10* (3), e0120201.
(107) Stubbs, A. C.; Martin, K. S.; Coeshott, C.; Skaates, S. V.; Kuritzkes, D. R.; Bellgrau, D.; Franzusoff, A.; Duke, R. C.; Wilson, C. C. Whole Recombinant Yeast Vaccine Activates Dendritic Cells and Elicits Protective Cell-Mediated Immunity. *Nat. Med. (N. Y., NY, U. S.)* **2001**, *7* (5), 625−629.
(108) Liang, B.; Li, L.; Mascin, M.; Liu, A. Construction of Xylose Dehydrogenase Displayed on the Surface of Bacteria Using Ice Nucleation Protein for Sensitive D-Xylose Detection. *Anal. Chem. (Washington, DC, U. S.)* **2012**, *84* (1), 275−282.
(109) Liang, B.; Li, L.; Tang, X.; Lang, Q.; Wang, H.; Li, F.; Shi, J.; Shen, W.; Palchetti, I.; Mascini, M.; Liu, A. Microbial Surface Display of Glucose Dehydrogenase for Amperometric Glucose Biosensor. *Biosens. Bioelectron.* **2013**, *45*, 19−24.
(110) Tang, X.; Liang, B.; Yi, T.; Manco, G.; IlariaPalchetti; Liu, A. Cell Surface Display of Organophosphorus Hydrolase for Sensitive Spectrophotometric Detection of P-Nitrophenol Substituted Organophosphates. *Enzyme Microb. Technol.* **2014**, *55*, 107−112.
(111) Tang, X.; Zhang, T.; Liang, B.; Han, D.; Zeng, L.; Zheng, C.; Li, T.; Wei, M.; Liu, A. Sensitive Electrochemical Microbial Biosensor for P-Nitrophenylorganophosphates Based on Electrode Modified with Cell Surface-Displayed Organophosphorus Hydrolase and Ordered Mesopore Carbons. *Biosens. Bioelectron.* **2014**, *60*, 137−142.
(112) Liu, A.; Lang, Q.; Liang, B.; Shi, J. Sensitive Detection of Maltose and Glucose Based on Dual Enzyme-Displayed Bacteria Electrochemical Biosensor. *Biosens. Bioelectron.* **2017**, *87*, 25−30.
(113) Wang, H.; Lang, Q.; Li, L.; Liang, B.; Tang, X.; Kong, L.; Mascini, M.; Liu, A. Yeast Surface Displaying Glucose Oxidase as Whole-Cell Biocatalyst: Construction, Characterization, and Its Electrochemical Glucose Sensing Application. *Anal. Chem. (Washington, DC, U. S.)* **2013**, *85* (12), 6107−6112.
(114) Petrenko, V. A.; Smith, G. P.; Gong, X.; Quinn, T. A Library of Organic Landscapes on Filamentous Phage. *Protein Eng., Des. Sel.* **1996**, *9* (9), 797−801.
(115) Lang, Q.; Wang, F.; Yin, L.; Liu, M.; Petrenko, V. A.; Liu, A. Specific Probe Selection from Landscape Phage Display Library and Its Application in Enzyme-Linked Immunosorbent Assay of Free Prostate-Specific Antigen. *Anal. Chem. (Washington, DC, U. S.)* **2014**, *86* (5), 2767−2774.
(116) Qi, H.; Wang, F.; Petrenko, V. A.; Liu, A. Peptide Microarray with Ligands at High Density Based on Symmetrical Carrier Landscape Phage for Detection of Cellulase. *Anal. Chem. (Washington, DC, U. S.)* **2014**, *86* (12), 5844−5850.
(117) Han, L.; Liu, P.; Petrenko, V. A.; Liu, A. A Label-Free Electrochemical Impedance Cytosensor Based on Specific Peptide-Fused Phage Selected from Landscape Phage Library. *Sci. Rep.* **2016**, *6*, 22199.
(118) Petrenko, V. A. Landscape Phage as a Molecular Recognition Interface for Detection Devices. *Microelectron. J.* **2008**, *39* (2), 202−207.
(119) Jones, K. E.; Patel, N. G.; Levy, M. A.; Storeygard, A.; Balk, D.; Gittleman, J. L.; Daszak, P. Global Trends in Emerging Infectious Diseases. *Nature* **2008**, *451* (7181), 990−993.
(120) Bisson, I.-A.; Ssebide, B. J.; Marra, P. P. Early Detection of Emerging Zoonotic Diseases with Animal Morbidity and Mortality Monitoring. *EcoHealth* **2015**, *12* (1), 98−103.
(121) Butler, D. How to Beat the next Ebola. *Nature* **2015**, *524* (7563), 22−25.
(122) Yamada, T.; Ogawa, V. A.; Freire, M. Policy: Security Spending Must Cover Disease Outbreaks. *Nature* **2016**, *533* (7601), 29−31.
(123) Hoyt, K.; Hatchett, R. Preparing for the next Zika. *Nat. Biotechnol.* **2016**, *34* (4), 384−386.

DOI: 10.1021/acsami.6b09263
ACS Appl. Mater. Interfaces 2016, 8, 30649−30664