



Improving antibody drug development using bionanotechnology

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Monoclonal antibodies are being used to treat a remarkable breadth of human disorders. Nevertheless, there are several key challenges at the earliest stages of antibody drug development that need to be addressed using simple and widely accessible methods, especially related to generating antibodies against membrane proteins and identifying antibody candidates with drug-like biophysical properties (high solubility and low viscosity). Here we highlight key bionanotechnologies for preparing functional and stable membrane proteins in diverse types of lipoparticles that are being used to improve antibody discovery and engineering efforts. We also highlight key bionanotechnologies for high-throughput and ultra-dilute screening of antibody biophysical properties during antibody discovery and optimization that are being used for identifying antibodies with superior combinations of *in vitro* (formulation) and *in vivo* (half-life) properties.

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Introduction

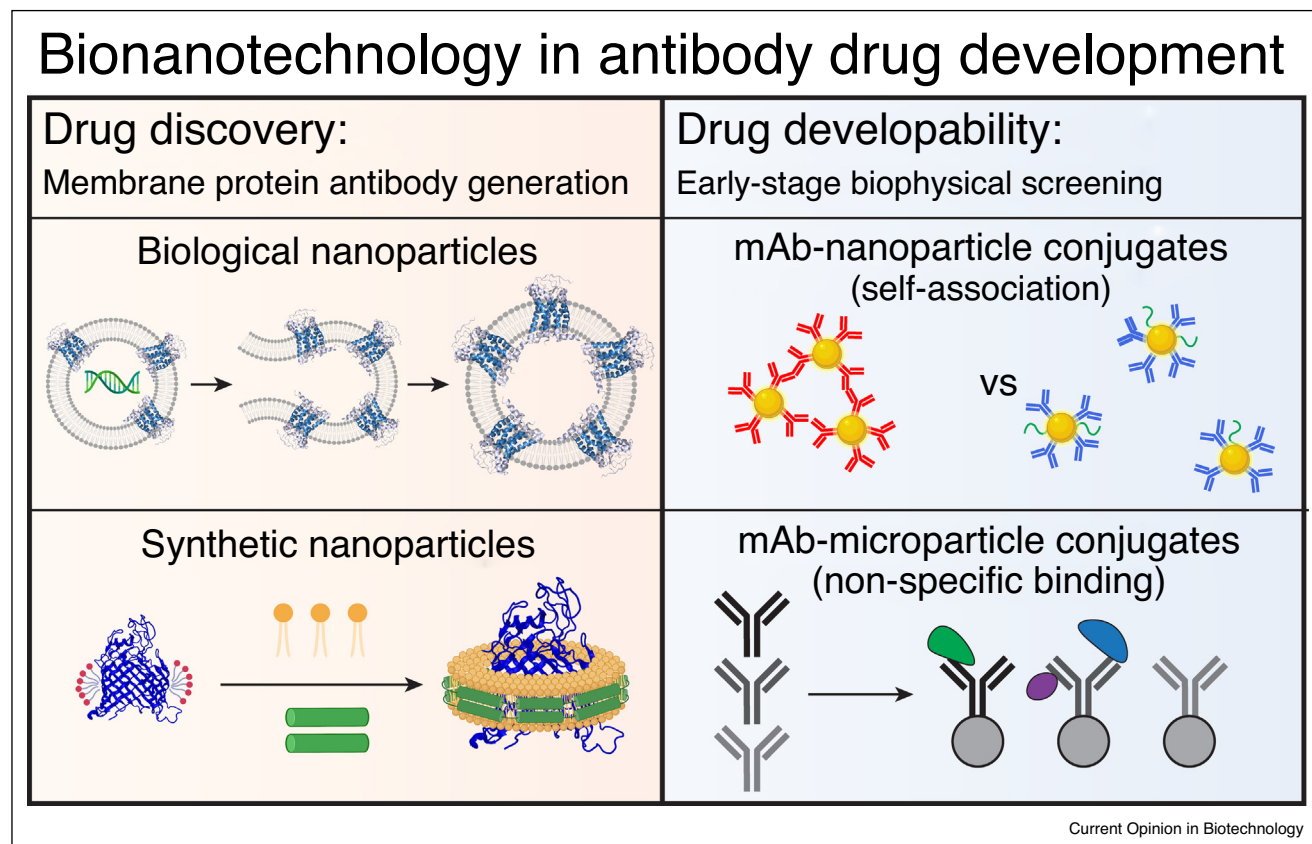
The success of antibodies as drugs is directly attributable to their unique combination of properties, including high affinity, potent effector functions, low toxicity, long half-life, and favorable biophysical properties (e.g. high specificity, stability and solubility). The FDA approval of >100 antibody drugs and related products and ongoing clinical trials for hundreds more has motivated a tremendous

amount of effort to improve the discovery and development of these molecules [1]. Despite great advances in this field, there are several key challenges that continue to limit the pace and success of antibody drug development [2–4]. In this review, we highlight two such challenges and illustrate how bionanotechnology holds great promise to address these challenges in ways that are increasingly simple and widely accessible (Figure 1).

Bionanotechnology for improved antibody drug discovery

One outstanding challenge for therapeutic antibody development is the discovery of antibodies against membrane proteins. As key mediators of myriad pathological conditions, membrane proteins represent a large fraction of promising therapeutic targets. As such, there is significant interest in establishing reliable methodologies to identify antibodies against these biomolecules. However, there are few approved antibody drugs specific for membrane proteins (<10) relative to the great number of unique membrane protein drug targets (thousands) [5–7]. The fundamental challenge for discovering antibodies against these targets is the ability to prepare membrane proteins in functional and soluble formats that can be used for either initial selection or secondary screening or both [8]. Unfortunately, membrane proteins are extremely difficult to produce as soluble antigens in stable, biologically relevant conformations. Transmembrane proteins, with highly hydrophobic midsections, are heavily reliant on an orderly lipid bilayer for stability and functionality, an environment that is difficult to recapitulate in a manner conducive to experimental selections of therapeutic antibodies. Several approaches have been reported for addressing this challenge, including DNA and RNA immunization [9,10], surfactant-solubilized membrane lysates [11] and whole cells [11–13] for generating antibodies against membrane proteins. However, these methods are limited by several common challenges, which are often linked to the low concentrations, purities and/or stabilities of membrane proteins either in lysates or displayed on whole cells. Moreover, DNA and RNA immunizations do not obviate the need for membrane protein reagents during secondary screening. Finally, *in vitro* antibody screening against whole cells is challenging due to the presence of many irrelevant protein antigens on the cell surface and the complexity of interfacing relatively large mammalian cells with yeast cells or even phage particles in a highly specific manner.

Figure 1



Overview of emerging bionanotechnologies that are improving antibody drug discovery and early-stage developability analysis.

One of the key challenges in antibody drug development is the generation of antibodies specific for membrane proteins given the difficulty in preparing soluble and functional versions of membrane proteins that can be used for immunization and *in vitro* antibody selections. Advances in generating diverse types of biological and synthetic lipoparticles displaying functional membrane proteins are simplifying the discovery of antibodies against a myriad of membrane proteins as well as diverse panels of epitopes and cross-species reactive epitopes. A second key challenge in antibody drug development is the assessment of antibody biophysical properties such as self-association and non-specific binding at the earliest stages of antibody drug discovery. This is particularly important because the complementarity-determining regions of antibodies, which govern antibody affinity and specificity, also mediate antibody self-association and non-specific binding. Advances in biophysical screening methods using bionanotechnologies is enabling the unusually large-scale screening of antibody self-association and non-specific binding in addition to affinity and specificity to identify antibodies with globally superior properties.

Encouragingly, several powerful bionanotechnology methods have emerged as promising general strategies for the controlled presentation of membrane proteins in a biologically relevant manner. These methods have facilitated the production of soluble, stabilized, and functional membrane protein antigens for use in immunizations and *in vitro* biopanning. Such methodologies have enabled both direct biological production of functional antigens (e.g. virus-like lipoparticles) as well as synthetic creation of functional antigens (e.g. nanodiscs and peptidiscs) (Figure 1).

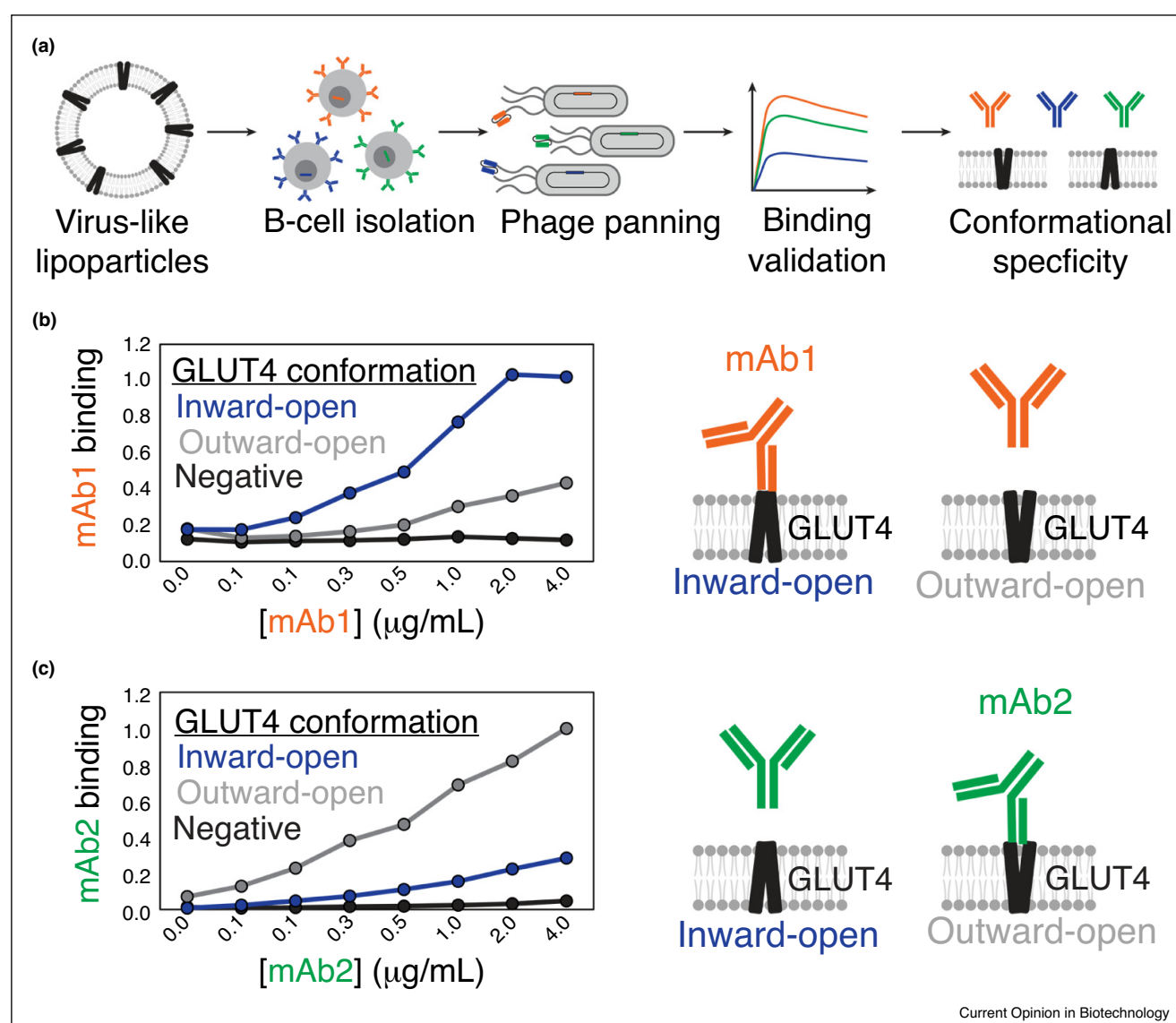
Virus-like lipoparticles are spheroidal assemblies (~150 nm in diameter) surrounded by intact plasma membranes [14,15]. These biological particles have been

produced using cellular systems overexpressing a desired cell surface antigen and Gag proteins. The Gag proteins localize to membranes and cause curvatures, which bud off as lipoparticles that can be purified via ultracentrifugation, PEG precipitation, or chromatographic separation [16,17]. Recently, these lipoparticles were employed for isolating antibodies against insulin-regulated glucose transporter (GLUT4) [18]. This transporter, containing 12 transmembrane spanning segments, is implicated in disease states including diabetes and obesity. Notably, virus-like lipoparticles were generated with human GLUT4 levels that were 10–100 times higher than those of conventional membrane preparations. For antibody discovery, human GLUT4 lipoparticles were first used for immunization, and then the resulting antibody

variable genes were cloned and reformatted as single-chain variable fragments (scFvs) for phage display screening (Figure 2a). Next, the scFv library was panned for binding to GLUT4 lipoparticles, and isolated scFvs were further evaluated by ELISA for binding to GLUT4 lipoparticles and lack of binding to control lipoparticles lacking GLUT4. Several antibody clones from unique sequence families were characterized and observed to bind cells overexpressing GLUT4. The antibodies failed to bind GLUT4 in western blot analysis, suggesting engagement of conformational GLUT4 epitopes.

Notably, several of the identified mAbs showed unique selectivity for GLUT4 conformational states (Figure 2b and c) and mAbs specific for the inward-open conformation also demonstrated inhibitory bioactivity, locking the transporters in an inactive state. More broadly, similar types of lipoparticles have been employed in several antibody discovery campaigns at various stages, including as biotinylated antigens for biosensor-based affinity measurements [9,19–21]. Overall, virus-like lipoparticles may offer several advantages over alternative membrane protein formulations in that they are highly immunogenic,

Figure 2



Discovery of conformation-specific antibodies against the membrane protein GLUT4 using virus-like lipoparticles.

(a) Presentation of the native conformations of GLUT4 (inward-open and outward-open) for immunization and B-cell isolation was achieved using virus-like lipoparticle technology. Phage panning with GLUT4 lipoparticles led to the isolation of several high affinity antibodies. **(b)–(c)** Two selected GLUT4 antibodies (mAb 1 and mAb 2) display conformational specificity for either the (b) inward-open (mAb 1) or (c) outward-open (mAb 2) conformations. The figure is adapted from a previous publication [18].

contain intact membrane lipid bilayers, and are relatively straightforward to produce.

Despite the many strengths of biological lipoparticles, there has also been much progress in generating different types of synthetic nanoparticles, which also contain lipids, for presenting membrane proteins for antibody discovery applications [22–27]. Initial efforts sought to solubilize intact membrane proteins via harsh detergents that often disturb antigen structure and stability. To obviate the need for such detergents, inspiration from high-density human lipoproteins led to the development of nanodiscs, colloidal assemblies of lipid bilayers with modified membrane scaffold proteins wrapped around the hydrophobic midsections [28]. Nanodiscs have been modified to present a wide range of complex antigens and were recently used to identify high affinity antibodies against a model transmembrane protein, human Nav1.7 ion channel [29^{*}]. A chimeric surrogate (VSD4-NavAb) containing a portion of the bacterial homolog NavAb was selected for the campaign, which enhances expression and stability. Structurally, VSD4-NavAb is a complex transmembrane protein, assembling into a homotetramer with a single ion pore flanked by four voltage-sensing domains. After expression of VSD4-NavAb in insect cells, the protein was solubilized with detergent and purified via chromatography. Addition of stoichiometric amounts of lipid and membrane scaffold protein resulted in displacement of detergent and the formation of nanodiscs, which were confirmed via SDS-PAGE after size-exclusion chromatography purification. Notably, biotinylated nanodiscs were employed for flow cytometry and fluorescence activated cell-sorting (FACS) applications, which showed improved signal relative to sorting with proteoliposome formulations. Encouragingly, the nanodiscs were successfully used to isolate high affinity B-cells via single-cell sorting of antigen-specific hybridoma cells after immunization of mice with VSD4-NavAb liposomes (Figure 3).

Despite successful applications of nanodiscs in antibody discovery campaigns, they are relatively challenging to produce, and often require optimization of molecular formulation components (e.g. lipid length and rigidity) on an antigen-specific basis [30]. To address this challenge, two alternative peptide-based formulations have been developed, namely Peptidisc and Salipro particles [31,32]. Peptidiscs are assemblies of transmembrane proteins stabilized by surrounding amphipathic bi-helical scaffold peptides [32], and, unlike nanodiscs, do not require additional lipids for stabilization. The scaffold peptide used for assembling Peptidiscs has proven amenable to the stabilization of multiple different antigens while requiring minimal optimization. Similarly, saposin-lipoprotein (Salipro) particles, which are based on a class of lipid-binding proteins containing amphipathic helices (saposins), facilitate the stabilization of transmembrane proteins in lipid nanoparticles [31]. In Salipro particles,

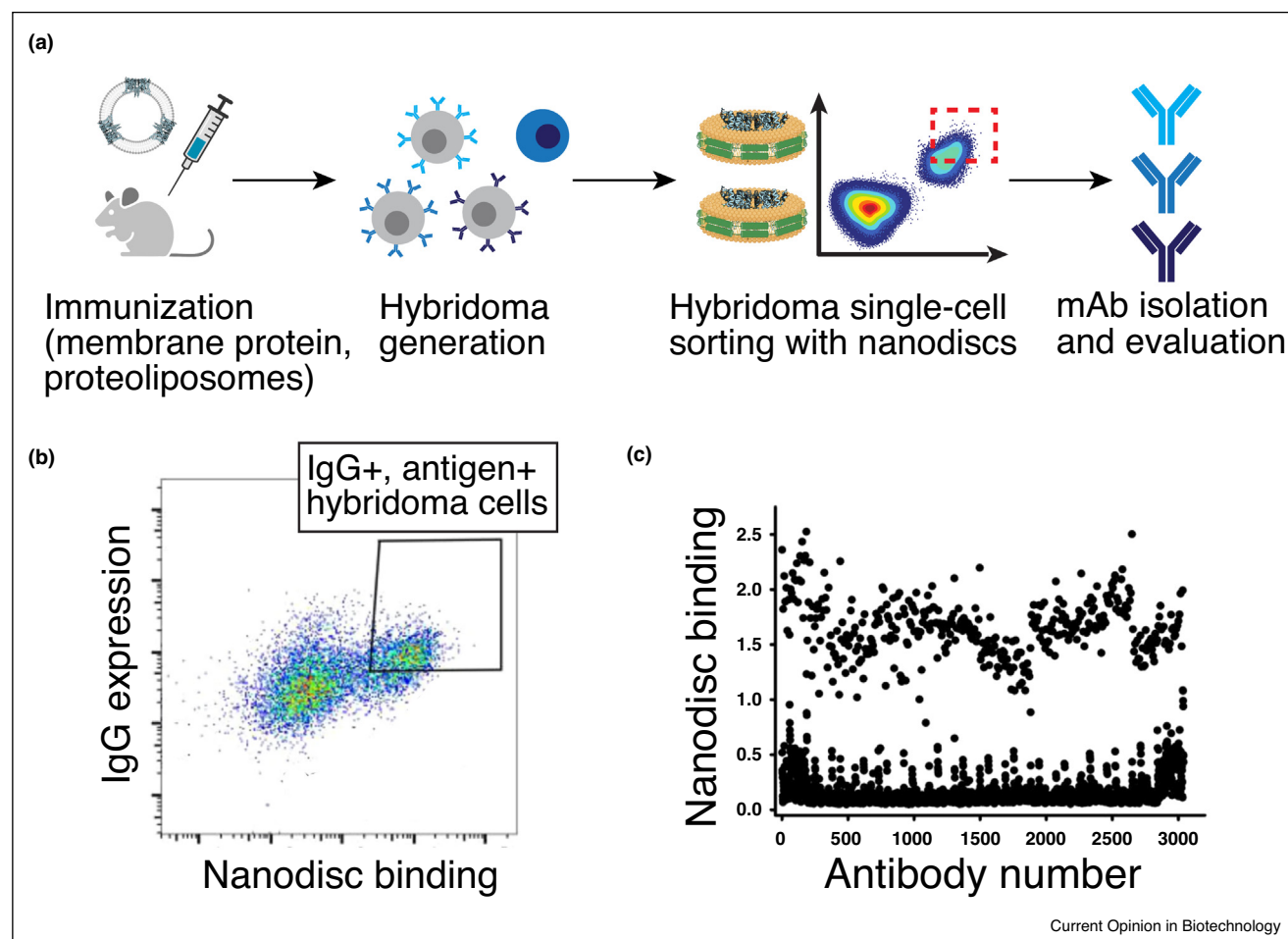
saposin-lipid complexes form a stabilizing belt around the hydrophobic region of transmembrane proteins. While the method of stabilization is similar to that for nanodiscs, the stabilizing components of Salipro particles are smaller and more flexible than nanodisc components, enabling natural adaptation of the number of stabilizing saposins to the size of complexed transmembrane proteins and lipids. This simplifies the optimization of Salipro particle production relative to nanodisc production. The Salipro technology has been used to stabilize multiple membrane proteins, including a bacterial peptide transporter and the HIV-1 spike protein, for structural and functional studies.

The generation of related synthetic lipoparticles has also been reported using polymers. In particular, styrene-maleic acid lipoparticles (SMALPs) have been validated for the synthesis of membrane protein formulations that are amenable to high-throughput antibody selections using cell sorting methods and exhibit improved thermostability over detergent-solubilized antigens [33]. These discoidal lipoparticles are generated from pores formed by the scaffold co-polymer (styrene-maleic acid) that surrounds target membrane protein within a native lipid bilayer. SMALPs have advantages over Salipro and Peptidisc particles because they do not require initial detergent extraction of membrane proteins and lack scaffold polypeptides, thereby reducing the risk of antibody isolation against non-target polypeptides [17]. Peptidisc, Salipro, and SMALP particles have not yet been reported as antigen formulations for the isolation of therapeutic antibodies against membrane proteins, but these unique particles hold great promise to improve such antibody generation and warrant future investigation.

Bionanotechnology for improved antibody developability analysis

A second outstanding challenge in the field of antibody drug development is the early-stage and ultra-dilute screening of antibody biophysical properties, especially antibody self-association because of its strong impact on concentrated antibody formulation properties such as viscosity and solubility [4,34–38]. There is intense interest in preparing antibody drugs as concentrated liquid formulations for subcutaneous administration, which increases patient compliance and quality of life and reduces costs associated with administration. However, antibody therapeutic candidates possess variable and difficult-to-predict properties in concentrated liquid formulations, including high viscosity, opalescence and aggregation, and these problems are typically discovered too late to address via selection of alternative candidates or protein engineering [39^{*},40^{*},41]. Therefore, there is a critical need for technologies that enable screening of antibody colloidal interactions at early stages of discovery, which are compatible with the large numbers of candidates (thousands) that are extremely dilute (<0.1 mg/mL)

Figure 3



Isolation of antibodies against the multi-pass membrane protein, VSD4-NavAb, using nanodiscs.

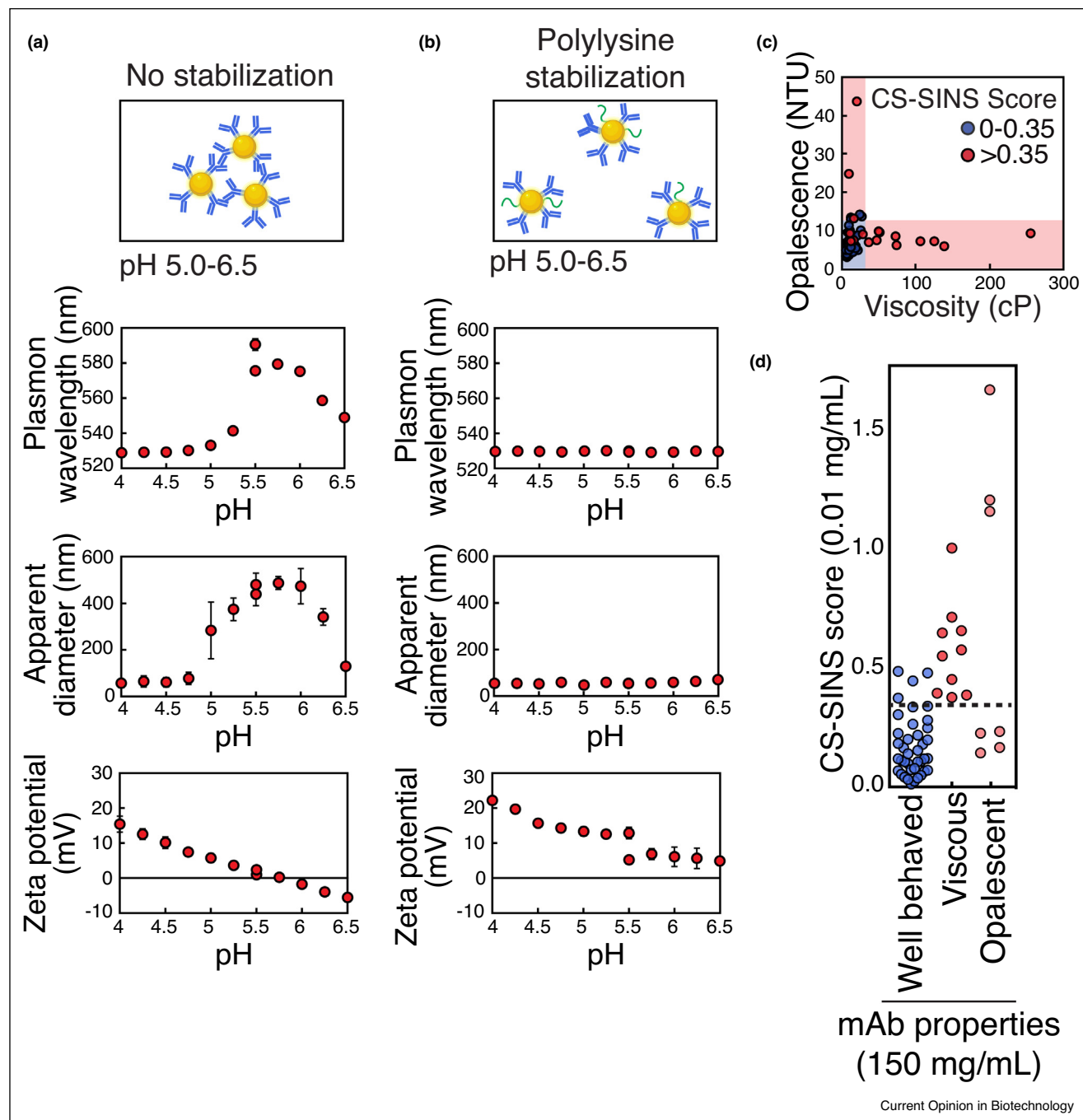
(a) Mice were immunized using the target membrane protein (VSD4-NavAb) prepared in proteoliposomes, hybridomas were generated, and then hybridomas were single-cell sorted using biotinylated nanodiscs presenting VSD4-NavAb. **(b)** Hybridoma cells were sorted to isolate single cells that both displayed IgG and bound nanodiscs presenting VSD4-NavAb. **(c)** Single-cell sorting of hybridoma cells using nanodiscs resulted in the selection of large numbers (>400) of antibodies with high binding activity, as judged by an ELISA assay with immobilized nanodiscs presenting VSD4-NavAb. The figure is adapted from a previous publication [29].

and only partially purified (e.g. one-step Protein A purification).

Encouragingly, bionanotechnology is enabling key advances in early-stage screening of antibody self-interactions. For example, a nanoparticle-based method has been reported for evaluating antibody self-association in physiological solution conditions, namely Affinity-Capture Self-Interaction Nanoparticle Spectroscopy (AC-SINS) [42]. This approach involves immobilization of anti-human IgG capture antibodies on gold nanoparticles (10–20 nm), and then capture of human mAbs at dilute concentrations (0.001–0.05 mg/mL). The colloidal interactions between the immunogold conjugates are evaluated in terms of the plasmon wavelength redshift via

measurement of the absorbance spectra, which can be performed using standard absorbance plate readers and 96-well or 384-well plates. This approach has been used for early stage screening to identify antibodies with superior solubilities [43] and viscosities [41]. In particular, this approach was used to evaluate the self-association of 87 mAbs generated against a common antigen using unpurified cell culture supernatants at ultra-dilute concentrations (0.001 mg/mL) [43]. Strikingly, the mAbs displayed a remarkably wide range of self-interactions, ranging from highly repulsive to highly attractive. Moreover, these self-interaction measurements were strongly predictive of the solubility of the human antibodies, as those mAbs with repulsive self-interactions displayed solubilities up to 200 mg/mL while those with strongly

Figure 4



Charge-stabilized affinity-capture nanoparticle spectroscopy (CS-SINS) enables ultra-dilute screening of antibody self-association for identifying candidates with low viscosity and opalescence in concentrated antibody formulations.

(a) Gold nanoparticles coated with anti-human Fc capture antibodies aggregate at weakly acidic pHs (e.g. pH ~5–6.5) and low ionic strengths (e.g. 10 mM histidine or acetate) because the zeta potential of the conjugates is low and crosses zero net charge around pH ~5.5. **(b)** Gold nanoparticles co-adsorbed with anti-human Fc capture antibodies and positively charged polymers (polylysine) do not aggregate at weakly acidic pH values and low ionic strengths because of the increased charge of the conjugates. **(c)** and **(d)** CS-SINS measurements of >0.35, which are measured at a mAb concentration of 0.01 mg/mL, display low risk for high viscosity (>30 cP) or high opalescence (>12 NTU) when formulated at 150 mg/mL. In (c) and (d), the solution conditions were pH 6 and 10 mM histidine. In (d), well-behaved mAbs refer to those that display both viscosity values <30 cP and opalescence values <12 NTU. The figure is adapted from a previous publication [39].

attractive self-interactions displayed solubilities below 10 mg/mL. This and other studies [44,45] demonstrate the significant potential of early-stage screening of antibody self-interactions to reduce the risk for suboptimal solution properties in concentrated antibody formulations.

Nevertheless, one key limitation of AC-SINS is that it is generally incompatible with common formulation conditions at acidic pH values (pH ~5–6.5) and low ionic strengths (e.g. 10 mM histidine or acetate). Therefore, there has been significant interest in adapting this assay to be compatible with common formulation conditions. A recent study demonstrated both the origin of the problem and a surprisingly simple solution [39]. After gold nanoparticles are coated with anti-human IgG capture antibodies, the investigators found that conjugates aggregated between pH ~5 and 6.5 regardless of the buffer (acetate or histidine), which was due to the zeta potential crossing from positive charge due to the highly positively charged antibodies to negative charge due to the negatively charged gold nanoparticles (Figure 4a). This led to the simple solution of co-adsorbing polylysine and capture antibodies to prepare gold-antibody conjugates that retained their positive charge in the key pH range of interest (pH ~5–6.5; Figure 4b). This modified assay, Charge-Stabilized Self-Interaction Nanoparticle Spectroscopy (CS-SINS), enabled ultra-dilute measurements of human mAb self-association (0.01 mg/mL), which was predictive of antibody solution properties at four orders of magnitude higher antibody concentrations (150 mg/mL). Antibodies with CS-SINS scores >0.35 displayed high probability of displaying abnormally high viscosity (>30 cP) or opalescence (>12 NTU; Figure 4c and d). This approach and other complementary approaches using functionalized nanoparticles with different charge and hydrophobic properties [46,47] hold great potential to identify antibody candidates with drug-like properties early in the discovery and optimization process.

In addition to the need to evaluate weakly attractive antibody self-association during early stages of drug development, it is also critical to evaluate weak non-specific colloidal interactions between antibodies and non-antigen molecules [48]. Antibodies with high levels of non-specific interactions have been linked to fast antibody clearance *in vivo* [49,50], which is due to several clearance mechanisms such as increased cellular internalization and degradation. There have been a wide range of proposed assays for evaluating antibody non-specific binding, including ELISAs [44,50,51], surface plasmon resonance [52], cross-interaction chromatography [53–55], and flow cytometry assays using various types of cells [56,57]. Despite the usefulness of these assays, they generally suffer from one or more common limitations, including low sensitivity for detecting non-affinity (non-specific) antibody interactions, low throughput and/or

lack of compatibility with existing soluble, full-length IgGs.

Recently, a magnetic particle-based assay, namely the PolySpecificity Particle (PSP) assay, has been developed for sensitively and rapidly evaluating antibody non-specific interactions [40]. The PSP assay involves capturing IgGs and other Fc-fusion proteins on micron-sized beads functionalized with Protein A, and then evaluating the binding of biotinylated reagents such as complex mixtures of proteins (e.g. soluble membrane proteins from mammalian cells) and proteins with diverse physico-chemical properties (e.g. ovalbumin) to immobilized IgGs via flow cytometry. The use of flow cytometry results in high signal-to-noise ratios and a strong ability to differentiate between antibodies with different levels of non-specific binding. Encouragingly, the PSP measurements are strongly correlated with previously reported levels of non-specific binding measured using a proprietary technology for displaying full-length IgGs on yeast [44], which is significant given that PSP uses soluble IgGs and is amenable to diverse types of antibodies and Fc-fusion proteins. Moreover, PSP is highly sensitive at ultra-dilute antibody concentrations (0.01–0.015 mg/mL) and simple to perform in a high-throughput manner using a standard flow cytometer with a microplate sampler [40]. This approach holds great potential for improving early assessment of antibody non-specific binding and generation of antibodies with reduced risk for abnormal pharmacokinetics.

Conclusions

The significant improvements in antibody discovery and developability analysis afforded by bionanotechnology raise several intriguing possibilities for future research and implementation. The advances in preparing soluble and functional versions of membrane proteins in various types of lipoparticles make it much simpler to perform sophisticated antibody selections using *in vitro* display technologies and generate a much greater array of antibody hits than has been possible previously. For example, routine generation of lipoparticles displaying functional membrane proteins, and the use of positive and negative antibody selections using phage or yeast surface display, would greatly simplify the selection of antibodies that recognize different membrane protein conformations, diverse epitopes and cross-species reactive epitopes. The advances in discovery-stage biophysical characterization of antibody self-association and non-specific binding now makes it possible to screen dramatically larger numbers of antibody candidates and antibody mutants during antibody discovery and optimization, which greatly simplifies the identification of antibodies with globally superior properties. This is particularly important because antibody selections against specific types of targets or specific epitopes, such as those that are hydrophobic and/or negatively charged, commonly results in

panels of antibodies with antigen-binding sites that are over enriched in hydrophobic and/or positively charged residues. These types of molecular features are key risk factors for poor developability properties and increases the need for extensive screening to identify rare antibody variants with drug-like biophysical properties. Finally, the importance of these advances in antibody developability analysis is expected to be even more important for the rapidly expanding class of multispecific antibodies and non-conventional biologics (e.g. antibody fragments, Fc fusion proteins, nanobodies and cytokines), which demand even greater emphasis on screening for drug-like biophysical properties given their unusual and non-natural formats that are much more prone to poor biophysical properties than conventional IgGs.

Conflict of interest statement

Nothing declared.

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Given his role as Guest Editor, Peter Tessier had no involvement in the peer review article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Ravi Kane.

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This study pioneers the methodology of Charge-Stabilized Interaction Nanoparticle Spectroscopy (CS-SINS), which enables early-stage antibody developability analysis of self-interactions at important antibody formulation conditions (pH ~6 and low ionic strength) and with ultra-dilute antibody concentrations (0.01 mg/mL). To address a key limitation of the parent technology (known as AC-SINS), the investigators co-adsorbed positively charged polymers and anti-human IgGs on the surface of gold nanoparticles to enhance electrostatic repulsion and thereby prevent aggregation that typically occurs at mildly acidic pH conditions (pH ~5-6.5). Overall, CS-SINS is a valuable demonstration of an applied nanotechnology to simplify early-stage, high-throughput antibody development analysis.

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