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Review

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Bacteriophages as Scaffolds for Bipartite Display: Designing Swiss Army Knives on a Nanoscale

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ABSTRACT: Bacteriophages have been exploited as cloning vectors and display vehicles for decades owing to their genetic and structural simplicity. In bipartite display setting, phage takes on the role of a handle to which two modules are attached, each endowing it with specific functionality, much like the Swiss army knife. This concept offers unprecedented potential for phage applications in nanobiotechnology. Here, we compare common phage display platforms and discuss approaches to simultaneously append two or more different (poly)peptides or synthetic compounds to phage coat using genetic fusions, chemical or enzymatic conjugations, and in vitro noncovalent decoration techniques. We also review current reports on design of phage frameworks to link multiple effectors, and their use in diverse scientific disciplines. Bipartite phage display had left its mark in development of biosensors, vaccines, and targeted delivery vehicles. Furthermore, multifunctionalized phages have been utilized to template assembly of inorganic materials and protein complexes, showing promise as scaffolds in material sciences and structural biology, respectively.

1. INTRODUCTION

Bacteriophages (phages for short) are a diverse group of viruses requiring bacterial hosts for propagation. Most are structurally fairly simple, comprising only a protein coat (termed the capsid) and encapsidated nucleic acid, lacking an outer lipid envelope.[1] The capsid typically self-assembles from a handful of different structural proteins that are arranged in a geometric pattern. In phage display, a foreign (poly)peptide is spliced to a structural phage protein and expressed on the viral surface.[2] As the gene encoding displayed (poly)peptide is harbored in the phage genome, genotype and phenotype are directly linked. This feature allows for simple identification of individual phage-displayed peptides via sequencing of the corresponding genome segment. The methodology is typically used to construct libraries of random peptides or protein variants from which individual clones with desired properties can be isolated through screening such as affinity selection against immobilized targets.[3,4] Moreover, display of peptides and proteins on virions also provides a platform for design of vaccines,[5] biosensors,[6] and delivery agents (e.g., for drug targeting or tumor imaging[7,8]). In this review, we focus on the most widely exploited vehicle in phage display, the filamentous coliphage (but discuss alternatives as well), and present approaches to simultaneously decorate the capsid with two different (poly)-peptides. We also discuss display of synthetic moieties by ways of chemical and enzymatic conjugation. *Bipartite* display has significantly broadened the utility of bacteriophages in life and material sciences, allowing simple and cost-effective production of designed bifunctional nanodimensional probes or targeted delivery vehicles for medical applications, and scaffolds for directed assembly of organic and inorganic materials. Here, we review recent advances in these fields.

2. PHAGE STRUCTURE AND BIOLOGY

This section contains basic information on the biology of phage species frequently used for phage display, all of which are *Escherichia coli* specific. It is by no means exhaustive and merely aims to facilitate the reader in understanding differences among display platforms.

2.1. Filamentous Phage. Filamentous coliphages (Ff phages) belong to the *Inoviridae* group and are characterized by circular ssDNA encapsidated by a tubular coat of roughly 6 nm in diameter and up to 1 μm in length.[9−11] The 6400-base wild-type (wt) genome harbors 9 tightly packed genes denoted by Roman numerals giving rise to 11 protein products (2 genes have internal in-frame translation starts) that are necessary for viral replication, export from host cell, or capsid formation. Phage proteins are labeled with p followed by a number (for example, p8 denotes protein product of gene VIII). Also present in the Ff genome is an intergenic region containing packaging signal directing virion assembly and replication origins for + and − strand synthesis. The capsid (Figure 1A) is composed mostly of gene VIII protein product (p8), also termed the major coat protein. In the wt-filamentous phage approximately 2700 copies of p8 enclose the ssDNA in a tube-like fashion. Four minor coat proteins (each in 5 copies) cap

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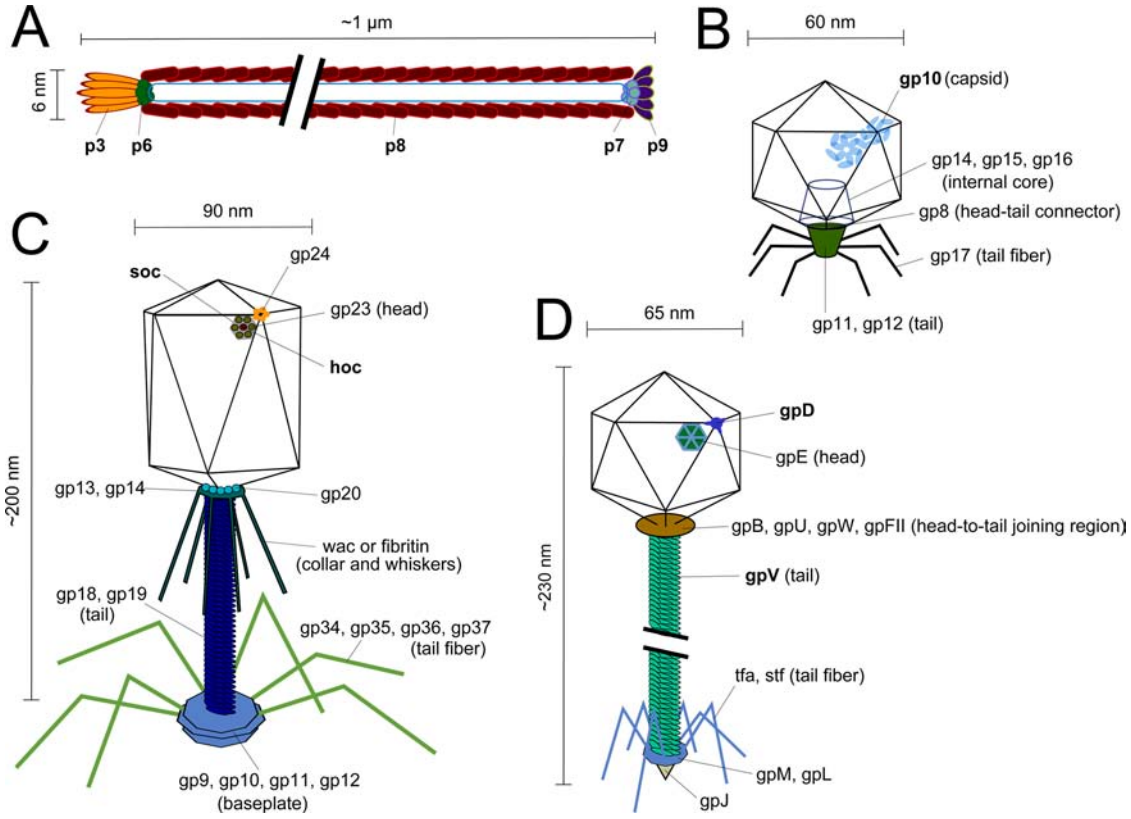

Figure 1. Architecture of phages used as display vehicles. Capsid proteins used as anchor proteins for phage display are labeled in bold. Note that drawings are not to scale. A. Filamentous phage (Ff). B. T7. C. T4. D. Lambda (λ).

the tube: p7 and p9 are found on the distal, and p6 and p3 on the proximal virion tip. Filamentous phages infect *E. coli* cells bearing F pili. The pilus acts as phage receptor to which virions bind via p3 whereas the host periplasmic protein TolA represents the coreceptor.[12] However, the exact post-receptor-binding events still await clarification.[13] During infection, the capsid is disassembled and coat proteins are retained in the plasma membrane. Once in the cytoplasm, phage ssDNA (the + strand) is converted to double-stranded replicative form (RF) that serves as a template for viral gene expression and ssDNA synthesis. Nascent ssDNA is stabilized by p5, preventing it from being converted to RF. Upon synthesis, coat proteins are directed to the plasma membrane, where they are incorporated into the nascent phage particles as they are released from the host cell through a channel formed by p1, p4, and p11. p7 and p9 directly interact with the packaging signal protruding from the p5-bound ssDNA and are required to initiate phage assembly. ssDNA is actively extruded from cells, whereby p5 is replaced by p8. Finally, p3 and p6 cap the virion and enable its release. Filamentous phages do not cause cell lysis and are continuously produced by infected cells. Filamentous phage structure and life cycle have been reviewed in more detail elsewhere.[9−11]

2.2. Lytic Phages. T7 bacteriophage is a member of *Podoviridae* group with icosahedral capsid of approximately 60 nm in diameter (Figure 1B).[14] The 40 kb genome in the linear dsDNA form contains some 55 closely packed genes flanked by terminal repeats. The capsid shell is formed by 415 copies of gene 10 protein (gp10), of which most represent the normal major capsid protein (gp10A), whereas about 5% represent C-terminally lengthened isoform termed gp10B resulting from translational shift. A stubby tail with 6 distal tail fibers is attached to one vertex of the head. T7 adheres to the lipopolysaccharide (LPS) core of the outer cell membrane with tail fibers upon which the inner capsule disintegrates. Its subunits presumably form a channel extending from phage tail all the way through the host cell inner membrane and allow for phage genomic DNA injection to the cytoplasm. Several phage genes are involved in repressing host transcription and evading host defense, and T7 RNA polymerase enables strong and specific transcription of phage genes encoding structural proteins. New virions are assembled directly in the cytoplasm and exit host cell upon holin- and lysozyme-mediated cell lysis.

T4, a *Myoviridae* group member, is also a tailed bacteriophage; however, its architecture is more complex (Figure 1C).[15] The quasi-icosahedral head (about 100 × 90 nm in size, making it one of the largest phages) houses a linear 170 kb dsDNA genome harboring some 280 open reading frames, and is mounted to a long contractile neck ending with a baseplate and tail fibers. On the opposite head joining side of the neck, a collar structure with 6 whiskers is situated. The head lattice consists of hexagonally arranged major capsid protein gp23 with gp24 corner protein pentamers at the vertices. Two dispensable proteins are symmetrically distributed over the head; 960 copies of Soc (small outer capsid protein) form a continuous mesh on the surface of gp23 hexamers, and Hoc (highly antigenic outer capsid protein) is found in the middle of each gp23 hexagon at 160 copies per head. Bacterial infection is a complex process mediated by tail fibers, whiskers, and baseplate, and is accompanied by numerous conformational changes of all substructures. As with T7 phage, viral DNA injection to the cytoplasm requires formation of a channel. T4 relies entirely on host RNA polymerase throughout the life cycle, and complex regulatory mechanisms direct T4 tran-

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scription and translation. DNA-filled heads of nascent virions are assembled independently from the tail and both subunits are finally joined to form infective phages that leave the host cell through lysis.

Lambda (λ) phage, belonging to the *Siphoviridae* group, is a temperate pathogen, meaning it can integrate its genome into the bacterial chromosome and thereby transmit its genetic information to bacterial progeny in a process known as the lysogenic cycle.[16] Infection is mediated by an outer membrane porin and the inner membrane mannose permease complex through which the phage injects its linear 48 kb dsDNA-genome. Upon activation of the lytic cycle, phage DNA is synthesized and genes required for assembly of novel virions and cell lysis are expressed. The icosahedral head measures 65 nm in diameter and is made from 415 gpE (major coat protein) copies (Figure 1D). Some 140 trimers of gpD (capsid decoration protein) stabilize the shell. The tail, made from gpV hexamer rings, is long and noncontractile. Similarly to T4, the dsDNA-containing head and the tail are assembled independently and subsequently joined.

3. CONSIDERATIONS ON CHOOSING THE DISPLAY PLATFORM

By far the most popular vehicle in phage display is the filamentous phage,[3,4] comprising closely related species M13, f1, and fd. Filamentous phage genome is small and easily amenable to genetic manipulation. Foreign inserts can be spliced directly to wt-structural genes, resulting in display on every copy of cognate protein product. Peptides and proteins are commonly displayed as N-terminal fusions to coat proteins p3 or p8 (Figure 1A; such vectors are dubbed type 3 or 8, respectively); however, all structural proteins can be used as display anchors. Display on C-termini has also been reported for p3,[17] p8,[18,19] p6,[20−23] and p9,[24] the latter with only limited success. While presentation of short peptides is readily tolerated, large proteins interfere with virion infectivity or prevent capsid assembly due to steric hindrance. The problem can be overcome in two ways.[3,4] First, another copy of the capsid protein gene is inserted in the nonessential region of the phage genome. Thus, one of the copies encodes the wt-protein and the other is the source of the recombinant fusion, giving rise to mosaic virion capsid. Such display systems are referred to as *cis* (e.g., type 33 or 88) throughout the paper. Alternatively, the recombinant copy of the anchor protein gene is supplied on a separate vector, such as plasmid or phagemid. Here, mosaic virions are assembled only upon infection of bacteria harboring the vector with a *helper* phage that acts as a delivery system for wt-phage genes (the process is known as *phage rescue*). These display systems are named *trans* (e.g., type 3 + 3 or 8 + 8). Not only do the *cis*/*trans* systems enable display of large proteins, both approaches allow for regulation of display valency. For example, when fused to wt p8 and p3, (poly)peptides are displayed in ∼3000 and 5 copies per virion, respectively, but with *cis*/*trans* systems the display valencies are up to several hundred and less than one, respectively.

A serious limitation of filamentous phage as display vehicle is that any (poly)peptide spliced to the capsid protein must be able to cross the plasma membrane in order to be integrated in the virion. Proteins containing long stretches of hydrophobic residues potentially acting as transmembrane stop transfer regions, or proteins that fold rapidly in the cytoplasm, may cause problems in this regard.[25] In addition, very large proteins fused to p8 that would significantly increase the overall diameter of the capsid are inefficiently displayed, because they hinder the passage of nascent virions through the 7 nm p4 exit pore.[10,26]

Alternatives to Ff phage used for phage display include lytic species T7,[27] T4,[28] and λ.[29] As progeny virions are released from bacteria by cell lysis, these platforms are faced neither with stringent limitations in size of displayed proteins (assuming steric hindrance is not an issue) nor with hydrophobicity of the polypeptide chain. Notably, even for combinatorial libraries of short peptides less sequence bias was observed with T7 compared to filamentous phage vectors.[30] However, with lytic phage species capsid expression of proteins containing intramolecular disulfide bridges may be problematic as reductive cytoplasmic environment prohibits SS-bond formation.[31] In contrast, filamentous phage-displayed proteins typically fold in the oxidative periplasmic space.

Probably the main reason for the widespread use of filamentous phage display platforms is the small genome size of Ff-species. Thus, phage vectors can be efficiently introduced into *E. coli* cells by simple electroporation. High transformation efficiency is especially warranted when constructing phage libraries as it is the most important factor determining library diversity. T7, λ, and especially T4 phages have much larger genomes that, when used as vectors, need to be packaged in vitro for subsequent transduction. This requires production of cellular extracts containing empty shells using an assembly compromised mutant virus. Alternatively, recombinant phages are produced by in vivo recombination using donor plasmids harboring foreign DNA.[32] In any case, formation of recombinant virions is laborious and rather inefficient. On the other hand, phages with large genomes can accept longer DNA inserts. For example, up to 24 kb of foreign DNA (more than one-half of wt genome) can be accommodated by λ phage by first removing nonessential loci. For λ phage, the limits of genome size for in vitro packaging are 78−105% that of the wt-λ genome.[33] In contrast, inserting foreign DNA into filamentous phage genome simply results in extended virions with additional p8 copies incorporated in the capsid. However, the longer the insert, the less efficiently the Ff progeny phages are produced in vivo. Moreover, with increasing length, virions become prone to breakage by shear forces. If capsid display of very large or multiple proteins is desired, *trans* systems may provide the solution.

T7 display relies on the major capsid protein gp10 (Figure 1B) whose C-terminus is exposed on the capsid surface. Two gp10 isoforms (shorter A and longer B) can self-assemble into head shell at various ratios, inspiring the idea of exploiting gp10 C-termini as display anchors.[27] Only relatively short peptides (up to 50 amino acids long) can be displayed at high valency (i.e., at up to 415 gp10 copies per virion), whereas large proteins are displayed at significantly lower levels and require a *trans* approach with native gp10 provided from a plasmid to support phage assembly.

In λ display, (poly)peptides have been mainly integrated into phage coat as fusions to C-termini of the head decorating protein gpD or major tail protein gpV (reviewed by Nicastro et al.;[29] Figure 1D). To avoid perturbation of the virion assembly, recombinant fusions that complement wt-capsid proteins are supplied in *trans*.[34] The *cis* approach based on conditional fusions has also been reported.[35] Here, a stop codon was inserted between the *gV* (gene V) part of the fusion and the

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downstream foreign gene, enabling production of mosaic tail virions in the suppressor host strain.

T4, although being a promising display platform, was not exploited as much as the aforementioned phages, likely due to its genetic and structural complexity. Most of the work on T4 display was centered on using decorative capsid proteins Hoc and Soc as anchors (reviewed by Gamkrelidze and Dabrowska;[28] Figure 1C). Fusions to one or both proteins can be harbored by the T4 genome[36] or be supplied in *trans* to Soc/Hoc-deficient phage (soc^-/hoc^-), whereby the fusion partner is incorporated into the head in vivo (i.e., during virion assembly in the infected cell[37]). Another strategy is to add isolated recombinant fusions to soc^-/hoc^- virions in vitro,[38] leading to spontaneous capsid decoration. Both *trans* and in vitro display strategies allow for display of large proteins or even protein complexes.[39] Since Hoc and Soc are dispensable capsid proteins, steric hindrance does not pose a problem in virion assembly, and display valency should be dependent on the size of the displayed protein.

Recently, a method for decorating filamentous phage noncovalently in vitro was also developed.[40] A net negative charge of the capsid was exploited to tether chemically synthesized peptides ending with 14 lysine residues (called "wrappers") along the length of the virion via electrostatic interactions.

4. BIPARTITE PHAGE DISPLAY: COMBINING TWO FUNCTIONS ON A SINGLE VIRION

Phage capsid is fairly tolerant to chemical[41−43] as well as genetic modifications (discussed above); typically, multiple capsid proteins were demonstrated to support surface display of in-frame fused (poly)peptides or synthetically conjugated compounds. The flexibility of display platforms encouraged attempts to construct phages simultaneously carrying two different heterologous proteins and/or coupled molecules. Here, phage takes on the role of a handle to which two modules are attached, each endowing it with specific functionality, much like the Swiss army knife. While combining diverse modules offers unprecedented potential for phage applications in bio- and nanotechnology, it is faced with certain limitations. Below, we review reports on bipartite phage display, discuss the design of phage vehicles, and innovative uses of recombinant virions.

4.1. Phage Biosensors. Among the first to recognize the potential of phage scaffolds to link two proteins were Light and Lerner.[44] Using a *trans* approach they created filamentous virions termed PhoPhabs simultaneously displaying antigen-binding antibody fragments (Fabs) and alkaline phosphatase (AP) as alternatives to labeled antibodies for enzyme-linked immunosorbent assay (ELISA). Bipartite phages were rescued from host bacteria doubly transformed with phagemid and plasmid vectors harboring *Fab-gIII* and *AP-gVIII* gene fusions, respectively. The probes could be produced with minimal cost and without the need for immunization because the recognition components (Fabs) were identified from semisynthetic (CDR-randomized) phage libraries. However, ELISA signal development required incubation of substrate-filled microtiter wells overnight, indicating extremely low valency of AP and/or Fab-display, likely due to the extreme size of both foreign proteins (approximately 50 kDa each) for filamentous phage display. To enhance sensitivity, recent phage biosensors rely on dual display of small proteins or peptides in order to achieve higher display valencies; single chain variable antibody fragments (scFv) are roughly half the size of Fab but preserve full binding activity, while short peptides make up for the low affinity with avidity effects as significantly higher display levels can be reached. Guo et al.[45] designed a phage probe as an antibody surrogate by displaying an anti-*Bacillus antracis* spore scFvs on the tip of filamentous virion capsid in a 3 + 3 format and fusing a gold-binding octapeptide to p8 of a helper phage. A highly sensitive phage immunoprobe for *B. antracis* spore detection was developed by first binding gold nanoparticles to p8-displayed peptide along the length of the virion and subsequent silver enhancement of the signal (absorbance read at 490 nm). As with PhoPhabs,[44] the physical separation of genes encoding fusion proteins on two separate vectors (i.e., phagemid plus plasmid/designed helper phage) allows for simple combination of various binding and detection components on phage scaffold without the need for laborious genetic engineering of each individual probe. Another example of *trans* modular phage biosensor design was reported by Rajaram et al.[46] They prepared filamentous virions concomitantly displaying a short streptavidin-binding peptide on p7 expressed from a helper plasmid, and phagemid-encoded UH-RA.21, a 28-amino-acid rheumatoid arthritis autoantigen, fused to p6. The helper plasmid lacked a packaging signal; therefore, recombinant phages were produced only upon transduction of host cells with phagemid virions. The dual display virions were used to enrich for anti-UH-RA.21 antibodies from patient sera in the course of screening. First, virions were preincubated with individual sera and the phage−antibody complexes were isolated using streptavidin-coated paramagnetic beads. Next, the complexes were captured on anti-human IgG-coated microtiter wells and detected with anti-phage Abs conjugated to horseradish peroxidase. An even simpler strategy for dual display was devised by Mohan and Weiss,[47] who managed to present two peptides on p8 by transforming bacterial host with a p8-encoding phagemid, subsequently transducing the cells with phage particles encapsidating a second p8-encoding phagemid using a high multiplicity of infection, and finally rescuing dual display virions with a helper phage. Although superinfection with the same phagemid is unstable in the long run, both copies are apparently retained by bacteria long enough to support incorporation of both encoded peptides in nascent virions.

Phages can also be used as frameworks for linking two ligands of a single target molecule. In this way binding is enhanced due to synergy of interaction, resulting in lower limit of detection when such a bidentate detection component is incorporated into a biosensor. For example, Mohan et al.[40] designed an electrochemical filamentous phage-based biosensor for extremely sensitive determination of prostate-specific membrane antigen (PSMA), a prostate cancer biomarker, in urine. One peptide ligand was displayed on p8 in a 8 + 8 format and the other was added in vitro in the form of synthetic fusion to oligolysine capsid "wrappers". Whereas the former was fixed to the phage surface, the latter was noncovalently bound, and could therefore reposition itself on the capsid until finding the optimal spot for bidentate binding.

Filamentous phage has proved suitable as a nanodimensional scaffold for chemical conjugation. In one application,[42] a biosensor for intracellular pH imaging was prepared by conjugation of two fluorophores, a pH-sensitive and a pH-insensitive one, to wt-capsid amine groups. Upon cell internalization as a result of endo/phagocytosis, doubly labeled virions allowed for accurate determination of pH in intracellular vesicles, both in vitro and in vivo (i.e., in macrophage cell line and in optically diffuse tissue in mice). The pH-insensitive

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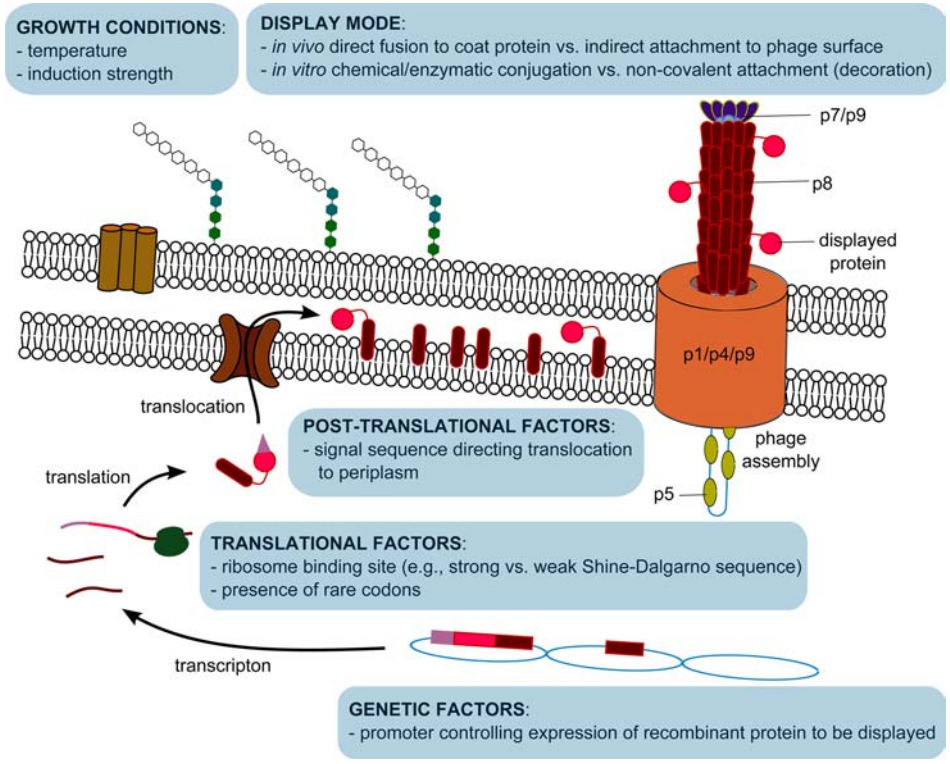

Figure 2. Some of the key factors to consider when displaying recombinant proteins on filamentous phage.

fluorophore acted as the reference for normalization of signal emitted by the pH-sensitive probe.

Reports on platforms other than the Ff phage for construction of biosensors are scarce. Recently, Tsuboyama and Maeda[48] described a prototype biosensor based on T7 virions simultaneously displaying a 15-amino-acid peptide (the N-terminus of RNase A, so-called S-tag) and green fluorescent protein (GFP) on the p10 anchor. Double display was achieved by expressing the p10-S-tag from a midvalency T7 phage vector, while the p10A-GFP fusion was provided from a plasmid. Successful parallel display of both (poly)peptides was demonstrated by fluorescent labeling of S-protein (i.e., RNase A lacking the 15 N-terminal residues) coated beads as determined by fluorescence microscopy. In another example, Pavoni et al.[49] constructed a λ phage concomitantly displaying anti-carcinoembryonic antigen (CEA) scFv on C-terminally truncated gpV, along with GFP or AP on gpD using a cis-type λ display vector. The recombinant phage had reduced infectivity compared to the wt one, likely due to display of a relatively large protein on the phage tail. Interestingly, the authors also noted that although plaques formed by scFv/GFP-phage and GFP-phage were equally bright, display levels of GFP in double-display virions were lower compared to the single-display counterparts, indicating lower efficiency of GFP incorporation in the virion head. Dual display of functional proteins on recombinant phages was confirmed by ELISA and indirect immunostaining (using anti-λ antibodies) of CEA-positive tumor cells in vitro and in vivo in tumor xenografts. Additionally, dual functionality of scFv/AP-phage was directly verified in dot blot and ELISA assays against recombinant CEA. The signals produced were, however, relatively weak, suggesting that display levels of AP were very low.

Similarly, we have been developing biosensors based on filamentous phage utilizing a 3 + 3/88 display platform (details to be published elsewhere). Specifically, anti-BSA scFv, expressed as a fusion to p3 from pIT2 phagemid,[50] served as a model affinity ligand; superfolder GFP (sfGFP[51]) was chosen as the reporter protein and was tethered to p8 expressed from a type 88 phage vector (f88−4[52]). Several different sfGFP/p8 expression cassettes were designed in an attempt to incorporate functional sfGFP, which folds rapidly in the cytoplasm, into the capsid at high levels. High-valency reporter display is essential as it conditions probe application in immunofluorescence techniques. We have focused on several factors such as peptide leader sequence for periplasmic tranlocation, mode of display (i.e., direct fusion vs indirect surface attachment), and conditions used for production of recombinant virions (Figure 2). Optimizations led to gradual improvement of display of both proteins on recombinant virions. Initially, the native p8 signal sequence of sfGFP-p8 fusion was changed to TorA peptide leader supporting periplasmic delivery of *folded* proteins through the Tat (twin-arginine translocation) pathway. Next, the genes encoding sfGFP and the recombinant p8 were decoupled to form a bicistronic expression cassette. In order to fasten the reporter protein to the virion surface, C-terminus of sfGFP and N-terminus of recombinant p8 were fused to the E- and K-coils,[53] respectively, which spontaneously self-assemble in a coiled coil structure. We have confirmed improvement in display levels of both proteins by phage ELISA and fluorescence microscopy. Last but not least, we also noticed that phage propagation conditions, especially induction strength of transgene expression and growth temperature, are of significant importance, affecting display valency as well as overall titer of produced virions. The display of both proteins and phage yield were best in the absence of the inductor (IPTG) and overnight growth of cultures at 30 °C. Drastically impaired culture growth rate in the presence of high IPTG concentrations suggested that bacterial host cells were

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significantly stressed when forcing expression from both phagemid and bacteriophage vectors.

4.2. Vaccine Design. In 1997 Malik and Perham[54] published a short paper describing display of two peptides on filamentous phage in a 8 + 88 format (i.e., fused to p8; one provided in *cis* from a phage vector along with the native major capsid protein, and the other supplied in *trans* from a phagemid vector). This proof-of-principle study was followed by reports on akin phage-based vaccines, exploiting high valency dual peptide display to elicit broad immune responses without the need for adjuvants. For example, de Berardinis et al.[55] designed a filamentous phage vaccine capable of evoking humoral as well as cellular immunity. They displayed two peptide epitopes of HIV1 reverse transcriptase fused to p8 of filamentous phage, one (presented by type I MHC) being responsible for provoking cytotoxic T lymphocyte (CTL) response, and the other (processed via type II MHC pathway) required for priming helper T cells (Th). When antigen presenting cells (APCs) were challenged with a 1:1 mixture of virions displaying single epitopes, only a relatively weak CTL response in vitro was noted. However, when APCs were exposed to virions coexpressing both epitopes, a much stronger CTL response was induced. This directly confirmed that CTLs are more efficiently activated by APCs that have processed both epitopes simultaneously, a feature made possible by concomitant delivery of both reverse transcriptase fragments. The vaccine was demonstrated to elicit potent CTL responses specific for the HIV-1 epitope in HLA-transgenic mice as well. The same group also reported on a highly immunogenic phage vaccine against cancer cells constructed in the same manner.[56] Here, one of the displayed peptides corresponded to fragments of melanoma-associated antigens (i.e., cancer/testis antigens or MAGE) and represented CTL epitopes, and the other (the HIV1 reverse transcriptase Th-epitope[55]) triggered helper T cell activation. The vaccine protected HLA-transgenic mice against MAGE tumors as demonstrated by 40% lower tumor incidence in vaccinated compared to unvaccinated animals after 80 days upon challenge with MAGE tumor cells. In yet another example of phage vaccines,[57] dual display was taken advantage of to target peptide epitopes preferentially to dendritic cells, the major class of APCs. A type 88 phage display vector was modified to a type 388 one, that is to say a single phage vector harboring a native and a recombinant (i.e., peptide-p8 fusion) gene *VIII* along with a gene encoding a scFv fused to p3. The anti-DEC-205 scFv enabled efficient DEC-205-receptor-mediated uptake of recombinant virions displaying an antigenic determinant of ovalbumin by dendritic cells. Moreover, the anti-DEC-205 virions induced maturation of dendritic cells without the need for other stimuli, leading to enhanced immune response. Again, the vaccine prompted strong CTL response and protected mice from experimental tumor growth (animals were challenged with murine melanoma B16 cells, stably transfected with ovalbumin).

In contrast to filamentous phage which is especially appropriate for high-valency display of peptide epitopes either genetically fused to the major capsid protein as discussed above or cross-linked to the capsid,[58] T4 platform supports display of full length immunogens at high copy numbers. Indeed, Wu et al.[59] designed a vaccine against the classical swine fever virus (CSFV) based on T4 Hoc/Soc bipartite display system, in which virions carried both the entire envelope structural protein of CSFV consisting of 371 amino acids fused to Soc (expressed from the phage genome) and the N-terminal outer membrane portion of the same protein, representing the major antigenic cluster, fused to Hoc (provided in *trans* from a plasmid). The vaccine elicited strong humoral response in vivo and anti-CSFV antibody titers were significantly higher in mice challenged with the bipartite vaccine compared to animals vaccinated with single display virions delivering either envelope structural protein isoform individually.

4.3. Cell Targeting. The ability to selectively target specific cell types is of central importance for achieving selective toxicity upon delivery of cytotoxic or antibiotic drugs as well as for high resolution tumor imaging. Phage vehicles, especially coupled with bipartite display strategies, have been successfully used in proof-of-principle experiments in both fields. It should be noted, though, that in contrast to phage vaccines the potential immunogenicity of phages when applied as delivery vehicles might seriously limit their practical use. The issue of immunogenicity is a well-known problem in phage therapy[60] as well as with eukaryotic vectors for gene therapy.[61] Anti-phage antibodies are more likely to lower efficiency of payload delivery upon multiple administrations; however, even naïve subjects may be preimmunized due to the omnipresence of phages in the environment.

4.3.1. Drug and Gene Delivery. Chloramphenicol is a highly effective bacteriostatic antibiotic; however, its use is limited by severe adverse effects such as hemolysis and bone marrow suppression. To overcome these issues, Yacoby et al.[62] prepared a chloramphenicol prodrug and conjugated it to filamentous phage capsid via esterase-cleavable linker using *N*-hydroxysuccinimide chemistry. The labeled phages relied on display of short peptides directed against *Staphylococcus aureus* (in a 88 format) to selectively deliver the antibiotic payload to bacteria. Alternatively, prodrug-labeled phages displaying an immunoglobulin-binding miniprotein (ZZ, an analogue of B-domain of staphylococcal protein A) fused to all five copies of protein 3 were incubated in anti-*S. aureus* antiserum. The bacteria were treated with targeting labeled phages in diluted serum as a source of esterases at titers at which the chloramphenicol concentration upon drug release would be below the minimal inhibitory concentration. Nevertheless, peptide- and antibody-bearing phages induced growth retardation of *S. aureus* in vitro as efficiently as the free antibiotic at 20- and 10-times higher concentrations, respectively. In contrast, *Streptococcus pyogenes* which showed comparable sensitivity to chloramphenicol was not affected by the same treatment. In a following study,[63] the drug delivery system was further improved by using a chloramphenicol-neomycin prodrug label, whereby the aminoglycoside antibiotic primarily served as solubility enhancer, enabling higher levels of prodrug conjugation. Moreover, the prodrug was tethered to phages displaying ZZ-domain *after* complexing with antiserum immunoglobulins by the EDC (1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide)) procedure, thereby also cross-linking antibodies to virions. This resulted in a staggering 20 000-fold improvement of potency over the free drug in vitro.

The same research group also designed phage-based delivery systems for targeting cytotoxic drug doxorubicin to cancer cells expressing growth factor receptors EGFR or ErbB2.[64] Here, p3-displayed ZZ-domain complexed with anti-ErbB2 (trastuzumab) or anti-EGFR monoclonal antibody (cetuximab) was exploited to deliver the drug to cancer cells in vitro. Doxorubicin was conjugated to p8 that was mutated at the N-terminus to display the tetrapeptide DFKG, a known cathepsin B cleavage motif. Thus, p8 was engineered for the

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purpose of controlled release. Upon binding to ErbB2 or EGFR cell receptors, phages were endocytosed, and the cytotoxic cargo was cleaved off in lysosomal compartments by cathepsin B. Again, the potency of phage-based prodrug was more than 1000-fold higher compared to that of free doxorubicin, demonstrating efficient and selective delivery.

A similar approach to tumor cell targeting using the filamentous phage platform was taken by Ghosh et al.[65] They designed a phage vector to concomitantly use *three* of the capsid proteins as anchors for peptide display. The heptapeptide fused to p3 served as a targeting component, selectively binding to SPARC (secreted protein, acidic and rich in cysteine), a tumor-associated glycoprotein found overexpressed in numerous metastatic cancers. Doxorubicin was chemically conjugated to DFK-displaying p8 for controlled release upon endocytosis. Moreover, a 15-amino-acid biotin acceptor peptide was fused to p9 and subsequently enzymatically biotinylated in vitro, which allowed for labeling the phage with streptavidin−AlexaFluor dye conjugate. In this way, the trifunctional phage enabled selective delivery of toxic payload to cancer cells and simultaneous cancer cell imaging, thus serving as a prototypical theranostic agent. The p9-biotin handle can, however, be functionalized with a number of different streptavidin-linked moieties, making the phage vector extremely versatile. To use p9 as an anchor protein, the authors first had to physically uncouple gene IX from gene VII. Namely, the gene VII stop codon overlaps with the start codon of gene IX, and the ribosome-binding site of gene IX is embedded in the coding region of gene VII. Therefore, N-terminal display on wt-p9 was not feasible before redesigning gene VII/IX region (except in *cis* or *trans* display systems with average display valencies of less than one).

The ability to permeate cell membrane and deliver the drug to intracellular compartments is of paramount importance in treatment of intracellular parasites such as *Chlamydia trachomatis*, a common sexually transmitted pathogen residing in specialized membrane-bound vacuoles (known as inclusions) of infected cells. To deliver intracellularly a short peptide mapping to the polymorphic membrane protein (Pmp) D (a highly conserved *Chlamydia* outer-membrane protein allegedly involved in the infection process), Bhattarai et al.[66] displayed it on p3 of filamentous phage along with the integrin-binding motif (RGD) containing peptide fused to p8. It was postulated that the PmpD peptide might competitively interfere with the infection process. The virions were taken up by HeLa and primary endocervical cells by integrin-mediated endocytosis. While virions displaying the RGD-peptide (but no PmpD peptide) were found in the cytoplasm surrounding the inclusions, the double display phages also translocated to inclusion lumen. When the host cells were treated with RGD/PmpD-displaying phages prior to or during the *C. trachomatis* infection, fewer inclusions were observed compared to cells that were incubated with wild-type and solely RGD-displaying phage, and the ones that did form were smaller. Hence, the RGD-motif peptide was responsible for intracellular access of the phage scaffold, and the PmpD peptide enabled distribution to inclusions and interfered with *C. trachomatis* infection, perhaps by blocking the acquisition of nutrients through the inclusion membrane.

Another intervention requiring intracellular delivery is gene therapy. While eukaryotic viral vectors enable highly efficient gene transfer, they suffer from low safety profile and high production costs. Phage vectors, on the other hand, can be rapidly produced at extremely low costs and show excellent stability, but are rather inefficient at transducing eukaryotic cells. Pioneering work in this field was performed by Larocca and co-workers by displaying cell-specific ligands on phagemid virions encapsidating mammalian expression cassettes.[67−70] Building on this idea, Zanghi et al.[34] hypothesized that by displaying a ubiquitinylation motif of hepatitis A virus (UBHA) on λ phage capsid, improved gene delivery to mammalian cells would be achieved due to proteasome involvement in head uncoating. In addition, a CD40-binding peptide (CD40bp) displayed on phage tail was expected to enhance uptake of viral vectors by macrophages. Lysogens harboring a mammalian expression cassette and deficient in gpD were transformed with two compatible plasmids. One harbored *gpD-UBHA* gene fusion, and the other contained a gene encoding CD40bp fused to the C-terminus of gpV. Upon heat induction of lysogens, virions displaying UBHA on all copies of gpD and CD40bp on roughly half of gpV copies were assembled. Luciferase was chosen as a reporter gene in order to easily monitor the efficiency of macrophage transduction. Virions composed of wt-capsid and the ones displaying only CD40bp were ineffective at mediating luciferase expression in targeted macrophages. UBHA-displaying vectors were, however, significantly more effective, even more so when both peptides were displayed simultaneously. Interestingly, λ phage genomic DNA levels were higher in lysates of cells transduced with CD40bp-displaying phages compared to phages displaying only UBHA, indicating that the ubiquitinylation motif enhanced gene transfer at a postinternalization stage, likely affecting proteasome-mediated uncoating or intracellular trafficking of internalized virions.

4.3.2. Biomedical Imaging. Phage probes seem especially suited for tumor imaging. Although much larger in comparison to labeled antibodies, phages exploit enhanced permeability of tumor blood vessels to gain access to solid tumors. Leaky blood vessels coupled with capsid display of tumor cell-targeting ligands provide the basis for specificity. On the other hand, due to the size of phage probes their use in immunocytochemistry is not anticipated as they are unlikely to produce the resolution achieved by much smaller antibodies.

The presence of functional groups of different reactivity on virion capsid allows *selective* attachment of multiple synthetic moieties to phage scaffold. Li et al.[43] exploited noncompeting amine and tyrosine conjugation to couple folic acid and fluorescent dyes, respectively, to filamentous phage. As coupling primarily took place on the functional groups of abundant major coat protein p8, a relatively large payload was introduced to phage capsid (some 200 and 400 molecules of folate and fluorescent dye per phage, respectively). Despite the relatively high degree of decoration, virions remained intact. Phage nanoparticles relied on folate receptor-mediated endocytosis for uptake of fluorescent payload by KB carcinoma cells. Internalization was assessed by fluorescent microscopy. Phage probes were found to give significantly higher signals compared to simple folate−fluorescein conjugates, presumably due to the avidity effect caused by a large number of receptor ligands in endocytosis.

In an early report on bifunctional phage probes for in vivo tumor targeting, Chen et al.[71] described a chimeric double display phage vector that they created by joining parts of a type 3 and a type 88 phage vector by restriction-ligation. They displayed an integrin receptor-binding peptide and a streptavidin-binding peptide fused to N-termini of p3 and p8,

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respectively. Phage probes were reacted with luminescent nanoscale quantum dots coated with streptavidin and targeted to α_V integrin expressing tumor-related blood vessels in mice xenografted with tumors. Phage probes were injected to mice intravenously; a few minutes later, animals were sacrificed and perfused through the heart, and organs were collected. Strong and well localized fluorescent signals were observed in deparaffinized tumor tissue slides using fluorescent microscopy. Here, the high signal-to-noise ratio can be explained by unrestrained access of phage probes to target vascular endothelium upon intravenous application and stringent removal of excess virions by perfusion. Nonvascular tumor-related antigens, however, are much more difficult to reach and their imaging requires a multistep targeting approach to deliver large quantities of *small* reporter groups to prebound tumor-specific probes. In one example, Newton et al.[72] constructed a bifunctional filamentous phage probe by displaying 5 copies of receptor-binding fragment of α-melanocyte-stimulating hormone on the virion tip and chemically conjugating biotin moieties along the length of the capsid. Melanoma-bearing mice were first injected intravenously with phage probes and, after 4 h with radioactive isotope ^{111}In, coordinately bound to diethylenetriaminepentaacetic acid-functionalized streptavidin. Phages were retained within melanoma and acted as anchors for isotope label, thereby enabling scintigraphic imaging of tumors. It was envisioned that the phage platform could be tailored to specifically target diverse cell types. Indeed, in a following study[73] a probe for prostate carcinoma imaging was designed based on a type 3 display filamentous phage clone expressing a short peptide homing to PC3 tumor cells that had previously been selected in vivo in mice.[74] Here, tumor imaging was further improved by taking advantage of the three-step pretargeting scheme. Mice with PC3 xenografted tumors were first injected intravenously with the biotinylated phages which were allowed to circulate for 4 h before a second injection of avidin was applied. Finally, after 24 additional hours mice received an injection of ^{111}In chelated to biotinylated 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid. Owing to the small molecular weight of the radiolabeled biotin, tumor uptake was enhanced while more efficient clearance was achieved due to extensive glomerular filtration, leading to significantly higher tumor to nontarget tissue signal ratio.

Even a single step (i.e., direct) tumor targeting can be efficient, provided that the probe is densely decorated with reporter groups. Filamentous phage is an ideal scaffold for high density display of *short* peptides; fusion of foreign peptides to every copy of p8 creates the so-called landscape phage[75] with display valency of several thousand. Ghosh et al.[76] constructed a phage probe displaying triglutamate peptides in a landscape format and a SPARC-binding peptide fused to p3. Triglutamates induced spontaneous assembly of stable magnetic iron oxide nanoparticles along the phage scaffold. Mice xenografted with SPARC-expressing prostate tumors were intravenously injected with MRI contrast probes and imaged with magnetic resonance scanner after 24 h. Iron uptake to tumors was much higher when phage probes decorated with magnetic nanoparticles were used compared to SPARC-binding peptides directly functionalized with the same contrast agent, therefore strongly (and specifically) reducing tumor MR signals. This work provided direct proof that in order to improve the contrast of MR images (or imaging in general), it is better to exploit carriers that deliver multiple contrast agents per receptor as opposed to the commonly used strategy of functionalizing each agent to multiple receptor ligands. Namely, low expression or accessibility of surface receptors might limit targeting efficiency and consequently payload delivery.

In yet another example of landscape phage imaging probes reported by the same group, Yi et al.[77] stabilized single-wall carbon nanotube (SWNT) fluorescent reporters by adhering them to phage displaying SWNT-binding peptide fused to p8. Concomitantly, peptide ligand of SPARC was directly displayed on p3, or alternatively, a prostate-specific membrane antigen-targeting antibody was attached indirectly to p3 (i.e., via genetically fused and subsequently enzymatically derivatized biotin acceptor peptide). Human xenograft prostate tumors were efficiently imaged in mice, taking advantage of specific targeting moiety and good penetration depth of near-infrared fluorescence signal of SWNT reporter.

4.4. Bipartite Display in Structural Biology and Material Sciences. Phage multifunctionalization can also be exploited for structural studies of protein complexes, wherein virions serve as carriers. To test the limits of the T4 display system, Li et al.[39] set out to assemble a large hetero-oligomeric protein complex, the 710 kDa anthrax toxin on the virion shell. Anthrax toxin is composed of two toxic components, lethal (LF) and edema factors (EF), attached to heptameric protective antigen (PA) forming a ring-shaped structure. PA-ring presumably acts as a membrane pore for intracellular delivery of LF and EF. Native anthrax toxin was sequentially reconstructed on T4 head in vitro by first saturating *hoc*$^-$/*soc*$^-$ phages with LF or its N-terminal domain (LFn) in the form of Hoc and Soc fusions, respectively, and subsequently incubating virions with a proteolytically activated form of recombinant PA, and later with recombinant EF. Interestingly, the LF-Hoc and LFn-Soc fusions bound to *hoc*$^-$/*soc*$^-$ T4 shell as efficiently as the native Hoc and Soc proteins, and even when used concomitantly occupied virtually all available binding sites without any steric interference. The authors estimated that 80% and 65% of LF-Hoc and LFn-Soc tethered PA heptamers, respectively, bound at least one EF subunit. Unprecedented display density (estimated to 229 complexes per virion) was achieved despite the extreme size of assembled macromolecular complex. Astonishingly, the combined weight of displayed complexes was more than 130 MDa, about 2.7 times the mass of *hoc*$^-$/*soc*$^-$ T4 shell. Nevertheless, phage capsid remained very stable. This study indicates the huge potential the T4 display platform holds in structural biology, for example, for stoichiometric analysis or structural studies by cryo-electron microscopy of large complexes reluctant to crystallize. An additional advantage of T4 display platform is that the shell can easily be decorated in vitro, thereby not restricting subunit production to *E. coli* expression system which is especially important when complex post-translational modifications are required.

More recently, Hess et al.[78] devised a way to display protein complexes on filamentous phage capsid as well. Preassembled pentamer of cholera toxin B subunits was enzymatically conjugated to p3 using sortase-catalyzed transpeptidation reaction. Sortases recognize the peptide motif LPXTG located near the C-terminus of a polypeptide, cleave it, and form a thioester-linked acyl-enzyme intermediate that is subsequently attacked by the nucleophilic N-terminal amino group of the second, oligoglycine-containing substrate, thus forming a new peptide bond. Initially, the authors attempted to display the sortase motif on the C-terminus of p3; however, this modification was not compatible with phage assembly. They

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solved the problem by inserting *in* p3 a 50-amino-acid sequence containing the sortase motif and flanked it with two cysteine residues that formed a disulfide bridge. Additionally, factor Xa cleavage sequence was integrated downstream of the sortase motif, enabling proteolytic loop cleavage and motif exposure, which greatly improved transpeptidation reaction efficiency. A similar strategy was later used to selectively label filamentous virions at opposite tips with synthetic peptide−oligonucleotide conjugates using p3 and p9 as anchors (here, the oligoglycine peptide fused to p9 served as the nucleophile acceptor). Finally, modified virions were interconnected through bridging oligonucleotides displaying complementarity to p3- and p9-linked DNA sequences, forming linear dimeric, trimeric, and tetrameric structures, dependent on displayed DNA. Importantly, a third orthogonal transpeptidation site was engineered on p8, providing a handle for attachment of additional functional moieties. In the future, such phage vectors may find use in assembly of highly complex multiphage structures containing protein or nucleic acid effectors.

In the field of material sciences, filamentous phages have often been exploited for their ability to self-assemble into an ordered structure and upon proper functionalization to template assembly of inorganic materials. In a proof-of-principle experiment, Huang et al.[79] displayed a streptavidin-binding peptide on p3 and a gold binding-peptide on p8 in a landscape format. By incubating bifunctional phages with gold nanoparticles, the latter arranged into an ordered unidimentional fashion along the virion length. Streptavidin-binding moiety on the virion tip allowed for specific orientation of gold nanocrystal arrays, forming more complex nanostructures, such as two arrays connected linearly or three arrays connected in a Y-shaped arrangement. Incidentally, the same research group also designed *circular* filamentous phage nanostructures by displaying a streptavidin-binding peptide and hexahistidine-tag on the opposite virion tips (fused to p3 and p9, respectively) and inducing cyclization with stoichiometric addition of streptavidin functionalized with Ni^{2+}-nitrilotriacetic acid groups.[80] Onto nanocrystal arrays gold from solution could be additionally deposited by selective reduction from $HAuCl_4$ in the presence of hydroxylamine, resulting in much thicker nanowires. The authors envisioned such metal nanowires to route electrical carriers to p3-templated nanostructures in a highly controlled way, improving connections on a molecular scale. Further developing this idea, they reported on construction of a high power Li-ion battery using phage-templated nanowires to guide assembly of percolated hybrid nanostructures composed of active and conductive materials.[81] Lithium metal foil served as the negative electrode, and assembled nanostructures represented the positive electrode. Specifically, tetraglutamate-displaying landscape filamentous phages were loaded with silver nanoparticles, and $FePO_4$ was subsequently deposited on nanocrystal arrays to generate nanowires. As the phages also displayed carbon nanotube-binding peptides fused to p3, incubation with single-walled carbon nanotubes (SWNT) resulted in formation of a well-dispersed mesh of $FePO_4$/SWNT hybrid nanostructures. Nanowires based on bifunctional virions increased electronic conductivity by improving electrical contacts between conductive and active materials as well as enhanced SWNT dispersion by preventing their aggregation.

Due to capsid stability and the ease by which it can be genetically or chemically modified to display specific ligands of biological molecules or peptide binders of inorganic materials, filamentous phage has been proposed as an alternative to DNA tethers for optical tweezers-based studies of bead-tethered single molecules.[82] Filamentous virion capsid was found to be stiffer than DNA, thereby more appropriate for communicating bead-applied forces to proteins of interest. Additional advantages of phage tethers include the ability to strictly define tether length (by exploiting concomitant display of ligands at proximal and distal virion tip) and that the protein of interest can directly be displayed on phage (i.e., the virion itself carries the protein and simultaneously serves as the mechanical tether). In a proof-of-principle experiment,[82] a bifunctional phage displaying streptavidin-binding peptide or chemically conjugated biotin at p3 and hexahistidine-tag at p9 was used to tether streptavidin-coated polystyrene microspheres to anti-His tag antibody-functionalized coverslip and elastic properties of filamentous virion were analyzed.

4.5. Improving and Advancing Phage Library Screening. Dual display is often harnessed in phage display libraries to introduce specific tags to viral capsid in addition to combinatorial peptides or protein variants with the aim of facilitating virion isolation, immobilization, and/or detection. Here, the *trans* display strategy is especially appropriate since it allows for multiple combinations of concomitantly displayed library (poly)peptides and tags by varying plasmid- or helper phage-encoded tags. In one example, Bonnycastle et al.[83] used a bacterial host strain harboring either a streptavidin- or fibrinogen-binding peptide-p8 fusion gene on a plasmid to label p3-displaying virions for direct capture of phages out of culture supernatants, thereby simplifying the isolation/enrichment process prior to ELISA assay. Løset et al.[84] expanded the molecular biology "tool box" of filamentous phage display by creating a range of helper phages, allowing expression of different tags (i.e., hexahistidine-, FLAG-, or Avi-tag (an enzymatic biotinylation sequence)) on p7, complementing p3- and p8-phagemid display. They applied the helper phages in isolation, immobilization, and detection of virions in mono- and polyclonal populations. Caberoy et al.[85] tagged a cDNA library displayed on p10B of T7 phage with either FLAG- or biotinylation-tag fused to p10A, supplied from a plasmid. This enabled them to use anti-FLAG antibodies and HRP-labeled streptavidin, respectively, in phage ELISA, which was of considerable interest since there are no commercial anti-T7 phage antibodies available in contrast to numerous equivalents to use in filamentous phage display.

There are other advantages of using tandem display when screening phage libraries. Malys et al.[36] constructed a T4 peptide display library exploiting Soc and Hoc proteins as anchors. Genes encoding random pentapeptides fused to dispensable coat proteins were introduced into T4 genome by means of homologous recombination. The library was screened against T4 terminase large subunit gp17 with the aim of identifying its interaction partners. Peptides mapping to 9 different T4 proteins were identified. A number of affinity selected clones displayed peptides homologous to different regions of a single presumed binding partner. It is likely that bipartite display (coupled with high display valency) enabled efficient enrichment of otherwise low affinity peptides due to synergistic binding and/or avidity effects. In contrast, Lamboy et al.[86] relied on double display to more reliably detect specific interactions of phage-displayed ligands. They inserted a tetrapeptide containing a lysine residue into the N-terminus of p8 of the commonly used helper phage M13K07. This served to neutralize the negatively charged filamentous capsid

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which was responsible for nonspecific adsorption of virions to high-pI protein targets. Markedly decreased nonspecific binding was observed in p3-displayed library screenings and phage ELISA assays, comparable to using synthetic oligolysine to wrap-neutralize the virions.

Recently, Rangel et al.[87] reported on a modification of filamentous phage vector that enabled screening of peptide display library against intracellular targets. The so-called internalizing phage (iPhage) library was created by displaying random peptides fused to p3, and penetratin, a 16-amino-acid cell penetrating peptide, fused to recombinant p8 provided in *cis* along with the native major capsid protein. Encouraged by confirmation of receptor-independent cell entry (a feature contingent on penetratin display) and the ability to direct internalized virions to mitochondria by concomitantly displaying cognate localization signal on p3, the authors attempted to identify peptides that would disrupt cellular viability. They panned the random iPhage library against live human Kaposi sarcoma KS1767 cells, isolated mitochondrial/endoplasmic reticulum-enriched fractions after 24 h, and recovered enriched phages by direct bacterial infection. After 3 selection rounds, a few clones were isolated and tested for their biological activity. One of the peptides, either displayed on iPhage or synthetically fused to penetratin, induced morphological alterations of cells, ultimately leading to cell death. Subsequent studies identified RPL29, a protein involved in 60S ribosomal subunit biogenesis, as the peptide's molecular target and found that the peptide activated numerous molecular pathways in mammalian cells, including apoptosis, autophagy, and necrosis.

5. CONCLUSIONS

Bacteriophages, especially filamentous phages, are structurally and genetically fairly simple viruses easily amenable to genetic manipulation of coat proteins. The robust structure of the shell tolerates extensive modifications at different sites. By choosing specific capsid proteins as anchors and exploiting the strategy of supplying recombinant coat proteins alongside the native ones (either in *cis* or in *trans*), one can strictly regulate valency and topology of the display system. Recently, chemical and enzymatic modifications, as well as noncovalent decoration techniques in vitro, have additionally extended the reach of phage display. Concomitant display of diverse (poly)peptides or conjugated molecules endows the virion with multiple functions, such as target recognition, cell entry, and signal generation, or provides contacts in electrical circuits, or templates inorganic material assembly. Owing to the immense versatility and relative ease of handling, phage display has found applications outside the traditional combinatorial library screenings, and became accepted by the scientific community working in the fields of material sciences, vaccine design, and structural biology.

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Notes

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