Orthogonal labeling of M13 minor capsid proteins with DNA to self-assemble end-to-end multi-phage structures

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Abstract

M13 bacteriophage has been used as a scaffold to organize materials for various applications. Building more complex multi-phage devices requires precise control of interactions between the M13 capsid proteins. Towards this end, we engineered a loop structure onto the pIII capsid protein of M13 bacteriophage to enable sortase-mediated labeling reactions for C-terminal display. Combining this with N-terminal sortase-mediated labeling, we thus created a phage scaffold that can be labeled orthogonally on three capsid proteins: the body and both ends. We show that covalent attachment of different DNA oligonucleotides at the ends of the new phage structure enables formation of multi-phage particles oriented in a specific order. These have potential as nanoscale scaffolds for multi–material devices.
The building of more complex materials requires construction of multi-phage scaffolds, but this has been hampered by the inability to freely manipulate the major capsid protein located in the body of phage and the four minor capsid proteins located at the ends of the phage (pIII, pVI, pVII, pIX) to form specific connections between different M13 particles. Streptavidin-based conjugates\textsuperscript{6–8} and leucine zippers\textsuperscript{9} have been explored to connect virions through the pIII, pVIII, or pIX proteins, but the resultant structures neither displayed a 1:1 stoichiometry - as streptavidin can bind up to four biotin molecules - nor did they allow precise control over structure length.\textsuperscript{9}

DNA hybridization is a commonly used strategy to establish nanoscale connections. It has been used to order spherical viruses\textsuperscript{10–11} and order gold nanoparticles into crystal lattices.\textsuperscript{12–13} Although these and polymer-based particles can be conjugated with DNA\textsuperscript{14–15}, the use of M13 offers two main advantages: high aspect ratio scaffolds and five proteins that may be engineered for different functions. Crosslinking individual M13 phage particles by means of DNA hybridization would have several advantages: first, a 1:1 stoichiometry with easier control over the number of phage coming together at a single connection; second, specificity and versatility, as the sequence of a DNA oligonucleotide can be modified to form new orthogonal complementary pairs; and third, reversible ligations, as DNA-DNA interactions can be disrupted by heat and reformed by cooling.

We accomplished specific labeling of the N-termini of pIII and pIX, with a variety of substituents using the sortase enzyme from \textit{Staphylococcus aureus} (SrtA\textsubscript{aureus}).\textsuperscript{7} Sortase-catalyzed transpeptidation reactions comprise two steps: initial recognition of an LPXTG motif placed near the C-terminus of a polypeptide which SrtA\textsubscript{aureus} cleaves after the threonine residue to form a thioester-linked acyl-enzyme intermediate. This is followed by a nucleophilic attack by the $\alpha$-amine of an oligoglycine (poly)peptide, which resolves the intermediate. Because the LPXTG motif-containing (poly)peptide can be conjugated beforehand with any substituent of choice (e.g., fluorophore), the final product is the protein of interest - in this case pIII or pIX – labeled at the N-terminus with that substituent. The SrtA\textsubscript{aureus} catalyzed reactions are orthogonal to \textit{Streptococcus pyogenes} sortase A (SrtA\textsubscript{pyogenes})-mediated labeling of pVIII, as the enzyme recognizes an LPXTA motif and the intermediate is resolved by an N-terminal double alanine nucleophile\textsuperscript{7, 16} instead of the (Gly)\textsubscript{n} preferred by SrtA\textsubscript{aureus}.

Here we describe the installation of a loop structure comprising the LPXTG sortase recognition motif on pIII to enable C-terminal display. Using an M13 construct containing three sortase labeling motifs within the same virion, we demonstrate orthogonal labeling of pIII, pVIII, and pIX proteins. Using this construct, we built end-to-end multi-phage structures in a specific order by labeling the pIII and pIX proteins with DNA and different fluorophores on the pVIII.

**Results and Discussion**

**C-terminal phage vector display of the sortase substrate motif**

We first examined whether we could display the LPXTG sortase-recognition motif at the C-terminus of the pIII, pVI, or pIX proteins. Although genetic engineering of the M13 phage genome (Supporting Note 1) yielded the desired modifications as confirmed by PCR (Supporting Fig. 1), they were incompatible with phage assembly.

We then engineered the N-terminus of pIII to display a 50 amino acid sequence comprised of an LPETG recognition motif for SrtA\textsubscript{aureus} flanked by two cysteines. When these cysteines engage in disulfide bond formation, they form a loop similar to that displayed by the subunit A of cholera toxin.\textsuperscript{17} Because proteolytic cleavage of the loop improves labeling
we inserted a linker followed by a Factor Xa protease cleavage site immediately downstream of the LPETG motif (Fig. 1a). We confirmed that sortase, pIII, pIX, and pVIII remained intact upon incubation with Factor Xa (data not shown). Thus, only the engineered pIII is a substrate for Factor Xa. This phage construct will be referred to hereafter as loopXa-pIII.

C-terminal sortase-mediated labeling of pIII

We labeled the loopXa-pIII phage construct at pIII with a GGGK(TAMRA) peptide using SrtA _aureus_ (Fig. 1b). Factor Xa was included in the reaction. We analyzed the samples by SDS-PAGE under both reducing and non-reducing conditions, followed by fluorescent imaging, and immunoblotting with an anti-pIII antibody. Only under non-reducing conditions and when all four reaction components were present did we observe a 60kDa fluorescent anti-pIII reactive protein (Fig. 1b), consistent with the presence of an intramolecular disulfide bond and loop formation on a single pIII molecule.

Sortase-mediated transpeptidation reactions afford attachment of a wide range of molecules to this loop structure, including a pre-assembled protein complex of ~58kDa (Supporting Note 2 and Supporting Fig. 2). Of note, all the (poly)peptides conjugated in this fashion will display an exposed C-terminus.

Orthogonal labeling of three phage capsid proteins

In a first attempt to establish end-to-end phage dimers, we tried to directly link the loopXa-pIII phage and a phage containing a pentaglycine motif at the N-terminus of its pIII (G5-pIII phage) via SrtA _aureus_. No dimers were observed after 24hrs of incubation and only ~3% of structures were dimeric after 60hrs of incubation (Supporting Note 3 and Supporting Fig. 3).

Given the slow kinetics of direct phage-phage fusion using SrtA _aureus_, we hypothesized that the loopXa and pentaglycine motifs on phage could be individually labeled with oligoglycine or LPXTG-based peptides before phage-phage fusions occur. With the ability to label pVIII orthogonally with SrtA _pyogenes_, we created a phage construct (hereafter referred to as triSrt) containing three sortaggable motifs: loopXa on pIII, (A)2 on pVIII, and G5HA on pIX (all at the N-terminus of the respective proteins). This combination enables selective labeling of three proteins on the same phage particle. The HA tag was added to pIX to extend its N-terminus and allow identification of the protein by immunoblots, as no antibodies are commercially available for pIX. We labeled each of these proteins in the triSrt construct with different fluorescent molecules (Fig. 2a) in a stepwise manner. First, pVIII was labeled with K(TAMRA)-LPETAA using SrtA _pyogenes_ with subsequent purification of the desired reaction product by PEG8000/NaCl precipitation. The resultant TAMRA-pVIII phage was then incubated with SrtA _aureus_, GGGK-Alexa647, K(FAM)-LPETGG, and Factor Xa for 5hrs at room temperature followed by PEG8000/NaCl precipitation. This precipitation allows purification of the labeled virions away from the other reaction components, including the side reaction product K(FAM)-LPETGGK-Alexa647 resultant from sortase-mediated fusion of the individual fluorescent peptides. Each reaction was analyzed by SDS-PAGE under non-reducing conditions followed by fluorescent imaging and immunoblot using anti-pIII and anti-HA antibodies (Fig. 2b). In the final product, we observed a TAMRA fluorescent ~10kDa protein compatible with the molecular weight of pVIII, an Alexa647 fluorescent and anti-pIII reactive 60kDa protein (Fig. 2b, lanes 4 and 6), plus a FAM-fluorescent and anti-HA (pIX) reactive ~10kDa protein (Fig. 2b, lanes 5 and 6).

Labeling of pIII and pIX with DNA

Because we can now functionalize the ends of the same phage particle orthogonally with different molecules, we sought to form phage trimers by DNA hybridization (Fig. 3a).
Thiolated and Cy5-labeled DNA oligonucleotides were conjugated to either a (maleimide)-LPETGG or GGGK(maleimide) peptides (Table S1). The resultant DNA-peptide adducts were purified by size exclusion chromatography and analyzed by MALDI-TOF mass-spectrometry. The product displayed a size consistent with (maleimide)-LPETGG (~700Da) and GGGK(maleimide) (~400Da) peptides fused to the DNA oligonucleotides (Supplementary Fig. 4a). These were also analyzed by TBE-Urea PAGE followed by fluorescent imaging (Supplementary Fig. 4b). Upon reaction with maleimide-peptides, we observed a shift in mobility of the DNA, and did not detect any unreacted DNA, suggesting that all DNA was conjugated to the peptide.

Using SrtA<aer</sub> and the triSrt phage, we attached DNA-peptides to pIII and to pIX forming three different phage constructs: DNA A-pIX phage, DNA B-pIII-DNA D-pIX phage, and DNA E-pIII phage (Fig. 3a). The reaction products were purified by PEG8000/NaCl precipitation. Free DNA-peptide co-precipitated with the phage, so an additional dialysis step was performed to remove it. The purified DNA-labeled phage was analyzed by SDS-PAGE under non-reducing conditions, followed by fluorescent imaging (Fig. 3b). Labeling of pIX with DNA A and DNA D (Fig. 3b, left panel) resulted in detection of Cy5-fluorescent 19kDa and 22kDa proteins, respectively. This is consistent with the predicted size of the DNA-pIX species. When pIII was labeled with DNA B and DNA E (Fig 3b, right panel), we detected Cy5-fluorescent 75 kDa and 80 kDa proteins, respectively. These sizes are consistent with those expected for the DNA-pIII species.

**Formation of ordered phage trimers**

We mixed equimolar amounts of the above DNA-labeled virions, followed by addition of the hybridizing oligonucleotides DNA C and DNA F in 10-fold excess over phage (Table S1 and Fig. 3a). The mixture was heated at 95°C and cooled to 20°C, thus allowing DNA to anneal and connect the phage particles. Atomic force microscopy (AFM) showed that this heating and cooling did not disrupt the integrity of the phage structure. Analysis of the annealed phage structure by AFM showed the existence of multi-phage structures of 2–3μm in length (Fig 3c and Supplementary Fig. 5). No structures corresponding to phage particles interacting with more than one phage at its end were detected, suggesting that the connections were indeed 1:1. We analyzed the phage population compiling a histogram of the lengths of observed structures (Fig. 3d and Supporting Fig. 5). For each treatment, at least 50 structures were measured. The length of a single phage is ~880nm. We thus assume that a structure <1μm represents a single phage, 1–2μm is two connected phage, 2–3μm is three connected phage, and >3μm is more than three connected phage. We observed that 52% of phage structures were 2–3μm. Structures longer than 3μm were observed rarely (5.8%), the longest observed structure being 4.70μm. In contrast, when DNA C and DNA F were omitted from the reaction, 95% of the observed phage structures were less than 1μm and no 2–3μm structures could be found. Dynamic light scattering (DLS) showed an increase in the distribution of the particle sizes. When DNA C and DNA F were absent, we observed a peak for objects with a radius of ~100nm, corresponding to phage monomers. The size of the particles in the main peak increased significantly (~1300nm) when DNA C and DNA F were added. Particles comprising this peak were compatible with trimer structures based on the structures observed by AFM (Fig. 3d). Because phage is filamentous and not spherical, the numerical value of the hydrodynamic radii is reported to demonstrate only relative changes in size.

To confirm that the observed multi-phage structures were indeed formed by DNA hybridization, we incubated them with restriction enzymes: AatII cleaves the annealed DNA structure between DNA A–C, AgeI cleaves the connections between DNA D–F (Fig. 3a). The samples were analyzed using AFM and DLS (Fig. 3d and Supporting Fig. 6). Upon
digestion with the individual enzymes, we observed a decrease in the structure length of the 2–3μm phage particles (12% for AatII, 3.3% for AgeI), with structures of 1–2μm in length being the most prevalent (46% for AatII, 62% for AgeI). This shift was consistent with the size distribution observed by DLS, where the peak for both AatII and AgeI digest shifted to ~500nm, corresponding to dimer phage structures. When the multi-phage preparation was incubated with both enzymes, we no longer observed phage structures of 2–3μm in length and the majority of the population was under 1μm (67%) (Fig. 3d and Supporting Fig. 6). These results were supported by DLS, where the peak of particle sizes decreased to ~200nm. We speculate that not all phage particles return to the monomeric form for reasons of steric hindrance: the phage structures themselves shielded the hybridized DNA from the restriction enzymes.

To ensure that the multi-phage structures were connected in the desired order, we fluorescently labeled the pVIII of the triSrt phage using SrtA_{pyogenes} with different fluorophores\textsuperscript{7}, followed by DNA labeling. This yielded the following phage particles: TAMRA-pVIII-DNA A-pIX, DNA B-pIII-FAM-pVIII-DNA D-pIX, and DNA E-pIII-Alexa647-pVIII. We mixed these phage in equimolar amounts with a 10-fold excess DNA C and F, and imaged them by fluorescence microscopy (Fig. 3e and Supporting Fig. 7). We observed multi-color filamentous structures connected in the expected order: TAMRA, FAM and Alexa647 (Fig. 3a, Fig. 3e and Supporting Fig. 7). In the absence of DNA, such connected multi-color filamentous structures were not observed and only single-colored filaments were present (Supporting Fig. 7).

**Conclusions**

Here we expand sortase-mediated labeling of M13 bacteriophage by engineering a loop onto pIII to enable C-terminal labeling. The insertion of a cleavable loop allows C-terminal exposure of the sortase motif LPXTG, and thus enables attachment of a substituted peptide or protein at that site via exposed Gly residues. Using this new structure, we attach a fluorophore and an oligomeric complex protein, neither of which could ever be displayed on the phage capsid genetically. Engineering of this loop onto pIII enables labeling orthogonal to the previously established N-terminal labeling method.\textsuperscript{7, 18} Thus, we created a new phage construct with the loop structure on pIII, a pentaglycine motif on pIX, and a double alanine motif on pVIII. Although this configuration should theoretically allow direct phage to phage conjugation, we found this to be an inefficient reaction, possibly for steric reasons, and therefore resorted to the use of complementary DNA crossbridges to achieve our goal. We demonstrated as a proof of concept that the minor capsid proteins of phage can be labeled with DNA and used to form specific connections between different phage particles. This reaction was more efficient, with over 50% of observed phage structures displaying the length of trimers. The precision of this strategy surpasses earlier accomplishments in which phage were linked using leucine zippers: heterodisperse multi-phage structures were obtained with mean lengths of 3–4.5μm (6–8 phage) and variability of length from monomers to longer than 20 phage.\textsuperscript{9}

The DNA modified phage as a scaffold building block not only allows better control over the structures that can be produced, but this strategy should be readily extendable to create much longer multimers by the proper choice of different DNA sequences. Our work sets the stage for building more complex multi-phage structures, such as multi-way junctions,\textsuperscript{19} or combinations with DNA origami structures\textsuperscript{10} with the potential to control positions in three dimensions.\textsuperscript{20} Attached DNA may also be used as a functional material sensitive to the environment such as pH,\textsuperscript{21} or bind substrates through the use of DNA aptamers,\textsuperscript{22–23} which extend the properties of the proteins or peptides displayed on the phage capsid, which has potential in biosensing applications.\textsuperscript{24}
The specific connection of phage particles, which we demonstrate, provides control of interactions between multiple materials at the nanoscale. Although the phage particles connected in this work were identical genetically, we attached different fluorophores to their pVIII body protein to establish that the requisite linkages were being formed in a predetermined order. In principle, the ability to pattern phage with different pVIII proteins enables self-assembly of junctions between materials and formation of multi-material axial nanowires or even circuits. This ability potentially allows for phage-based devices where configuration and the proximity of materials are critical including transistor- and diode-based electronic devices.25–26

Methods

Phage Engineering

The oligonucleotides used in engineering phage are shown in Table S2. LoopXa-pIII phage was constructed from an M13KE vector (New England Biolabs). The vector was digested with Acc65I and EagI. The annealed oligonucleotides pIIILoop-C and pIIILoop-NC were annealed and ligated into the digested vector. The Factor Xa recognition site was introduced by mutagenesis using the Quik II Site-Directed Mutagenesis kit (Stratagene) with oligonucleotides pIIILoopXaTop and pIIILoopXaBottom. The p9G5HA vector phage construct served as template for the creating the triSrt phage. The loop containing the Factor Xa recognition site was installed on pIII as described above. Two alanine codons were introduced at the 5′ end of pVIII using PstI and BamHI restriction enzymes and the annealed pVIII-AA-C and pVIII-AA-NC oligonucleotides. The phage constructs were transformed, plated, and amplified as described.7

Sortase-mediated reactions

Sortase reactions were performed as indicated in the figures. A typical sortase reaction for labeling LoopXa-pIII phage consisted of 160nM phage, 30μM SrtAaureus, 230nM Factor Xa, 100μM GGGK(TAMRA) or G3 fused to the N-terminus of the B subunit of cholera toxin (G3-CtxB), and 10mM CaCl2 in TBS (25mM Tris, pH 7.0–7.4, and 150mM NaCl) incubated for 5hrs at room temperature. The concentration reported for G3-CtxB is the monomer concentration. The sortase labeling reactions with GGGK(TAMRA) were monitored by SDS-PAGE under reducing and non-reducing conditions followed by fluorescent imaging and immunoblot with an anti-pIII antibody (New England Biolabs). The CtxB labeling reactions were analyzed by SDS-PAGE in non-reducing conditions followed by immunoblot using an anti-pIII and anti-CtxB antibody (GenWay Biotech).

Typical conditions for labeling the pVIII of the triSrt phage were 160nM phage, 40μM SrtApyogenes, and 200μM fluorophore conjugated LPETAA peptide incubated for 3hrs at room temperature followed by PEG8000/NaCl precipitation.7 The end labeling reactions of pIII and pIX consisted of 160nM phage, 30μM SrtAaureus, 230nM Factor Xa, and 100μM of fluorescent peptide or 50μM of DNA peptide in 10mM CaCl2 incubated for 5hrs at room temperature followed by PEG8000/NaCl precipitation. For the DNA-phage reactions, additional purification was performed by dialysis against water with a 1MDa molecular weight cut-off (Spectrum Labs), followed by another round of PEG8000/NaCl precipitation to purify and concentrate the samples.

DNA peptide conjugation

The DNA oligonucleotides attached to the ends of phage are shown in Table S1. The thiol group on the DNA oligonucleotides was activated overnight with 0.1M DTT in PBS at 37°C. The DNA was then purified from excess DTT on a NAP5 column (GE Healthcare) and eluted in water. The solution was dried and resuspended in PBS. (maleimide)-LPETGG

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or GGGK(maleimide) peptide in PBS was added in 2:1 molar excess of the activated DNA and reacted for 5hrs at 37°C. In order to deactivate the excess maleimide, DTT was added to the mixture to give a concentration of 0.1M DTT and incubated at 37°C for 15min. The excess DTT and peptide was removed by purifying the reaction on a NAP5 column. The DNA-peptide was dried under vacuum and resuspended in TBS. The concentration of the DNA-peptide was determined by UV-vis spectrometry using the absorbance at 260nm. DNA-peptides were analyzed by a Micromass microMX MALDI with a pulsed 337nm nitrogen laser. Spectra were acquired in positive ion, linear mode with a mass range of 2–30kDa.

**Atomic Force Microscopy and Dynamic Light Scattering**

The three DNA labeled phage were mixed together at 7·10^{13} pfu/mL in water. Hybridizing oligonucleotides DNA C and F were added in 10-fold molar excess. The reactions were heated to 95°C for 5 minutes and cooled down to 20°C at 0.5°C per minute. For restriction enzyme digestion the phage were resuspended in NEB Buffer 4 (50mM postassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM DTT, pH 7.9), and incubated at 37°C for 3hrs. We verified that the DTT in the NEB buffer did not disrupt the LoopXa-pIII structure by exposing LoopXa-pIII phage with Factor Xa to the buffer. We analyzed the reactions by SDS-PAGE followed by immunoblot with an anti-pIII antibody and estimated by densitometry that 10% of the LoopXa-pIII structures were disrupted, which represents only 1 pIII molecule for every two phage suggesting this did not significantly affect the connections.

To visualize the samples by AFM, phage preparations were diluted in water to a concentration of 2·10^{11} pfu/mL. 90 μL of the phage solution was deposited on a freshly cleaved mica disc. AFM images were captured on a Nanoscope IV (Digital Instruments) in air using tapping mode. The tips had spring constants of 20–100N/m driven near their resonant frequency of 200–400kHz (MikroMasch). The AFM images were analyzed and processed using Gwyddion. The histograms were collected by measuring the length of all phage events observed in seven 20μm × 20μm areas.

DLS measurements were obtained with a DynaPro NanoStar (Wyatt Technology). Phage mixtures in NEB buffer 4 were diluted to 1·10^{13} pfu/mL in water. Samples from each experiment were measured 20 times and the results were averaged by cumulant analysis.

**Fluorescence Microscopy**

The phage samples were diluted to 6·10^{11} pfu/mL in water and 300μL were deposited and dried on a glass cover slip. The samples were imaged using an inverted DeltaVision microscope equipped with an epifluorescent illumination module - 488 nm laser (FAM - 488 nm) and solid state illumination (TAMRA - 543nm and Alexa647), an oil immersion 100X objective (N.A.=1.40, 100x, Olympus) and Photometrics CoolSNAP HQ camera. All images were processed using ImageJ program (National Institutes of Health).

**Miscellaneous**

Expression and purification of SrtAp_yogenes, SrtA_{aureus} and G_{3}-CtxB were performed as described. The LoopXa-pIII reactions were analyzed on 10% Laemmli SDS-PAGE gels. The plX-DNA reactions were analyzed on a 16% Tricine-SDS PAGE gel, and the DNA-peptide conjugation reactions were analyzed on a 10% TBE-Urea PAGE gel (Life Technologies). All fluorescent gel images were collected on a Typhoon Trio (GE Healthcare). The GGGK(TAMRA), K(FAM)-LPETGG, GGGK(maleimide), (maleimide)-LPETGG, K(TAMRA)-LPETAA, and K(FAM)-LPETAA peptides were obtained from the Swanson Biotechnology Center. For mass-spectrometry, the protein bands of interest were
excised, subjected to protease digestion, and analyzed by electrospray ionization tandem mass-spectrometry (MS/MS).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
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<td>DLS</td>
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<td>FAM</td>
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<td>TAMRA</td>
<td>tetramethylrhodamine</td>
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**References**


Figure 1. Labeling of loop-pIII

Schematic for C-terminal labeling using the loop structure (a). LoopXa-pIII phage was incubated with SrtA\textsubscript{aureus}, Factor Xa, and GGGK(TAMRA) (b). The reactions were monitored by SDS-PAGE under reducing and non-reducing conditions followed by fluorescent imaging and immunoblotting with an anti-pIII antibody. The molecular weight markers are shown on the left.
Figure 2. Orthogonal labeling of phage with three fluorophores
Schematic representation of the strategy used for triple labeling of a single phage particle (a). TriSrt phage (lane 1) was incubated with SrtA_{pyogenes} and K(TAMRA)-LPETAA and purified by PEG8000/NaCl precipitation (lane 2). The TAMRA-pVIII labeled triSrt phage was incubated with Factor Xa, SrtA_{aureus}, FAM-LPETGG, and/or G3-Alexa647, and purified. These reactions were monitored by SDS-PAGE under non-reducing conditions, followed by fluorescent imaging and immunoblotting with an anti-pIII or anti-HA antibody (b). The molecular weight markers are indicated on the left.
Figure 3. Building phage by DNA hybridization
Scheme of the multi-phage final structure upon DNA hybridization (a). TriSrt Phage was incubated with DNA-peptides, SrtA\textsubscript{aureus} and purified by PEG8000/NaCl precipitation. The reactions were monitored by SDS-PAGE under non-reducing conditions, followed by fluorescent imaging (b). The samples with DNA-peptide alone had a concentration of 650nM instead of 50\mu M. The molecular weight markers are shown on the left. Phage were linked (see Methods for details) and imaged by atomic force microscopy (c). The length of the phage structures were measured and collected in a histogram and analyzed by dynamic light scattering (d). Fluorescently labeled phage were connected and imaged by fluorescent microscopy (e).