Phage Assembly Using APTES-Conjugation of Major Coat p8 Protein for Possible Scaffolds

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SYNOPSIS

Filamentous phages have been in the limelight as a new type of nanomaterial. In this study, genetically and chemically modified fd phage was used to generate a biomimetic phage self-assembly product. Positively charged fd phage (p8-SSG) was engineered by conjugating 3-aminopropyltriethoxysilane (APTES) to hydroxyl groups of two serine amino acid residues introduced at the N-terminus of major coat protein, p8. In particular, formation of a phage network was controlled by changing mixed ratios between wild type fd phage and APTES conjugated fd-SSG phage. Assembled phages showed unique bundle and network like structures. The bacteriophage based self-assembly approach illustrated in this study might contribute to the design of three dimensional microporous structures. In this work, we demonstrated that the positively charged APTES conjugated fd-SSG phages can assemble into microstructures when they are exposed to negatively charged wild-type fd phages through electrostatic interaction. In summary, since we can control the phage self-assembly process in order to obtain bundle or network like structures and since they can be functionalized by means of chemical or genetic modifications, bacteriophages are good candidates for use as bio-compatible scaffolds. Such new type of phage-based artificial 3D architectures can be applied in tuning of cellular structures and functions for tissue engineering studies.

Key Words: filamentous fd phage; self assembly; bio-compatible scaffolds; 3-Aminopropyltriethoxysilane (APTES); tissue engineering
INTRODUCTION

Recently, M13 filamentous phages have served as multifunctional nanoscale building blocks for the organization of materials. They demonstrated a potential use in high-performance memory, computing devices, energy storage materials, and tissue engineering applications\textsuperscript{1-6}. The functional versatility of fd phages is determined by their shape, charge and peptide display ability, all of which can be changed through chemical or genetic modifications. The fd phage is a rigid helical rod with -1 μm length and -7 nm diameter\textsuperscript{7,8} and consists of -2,700 copies of a major coat protein called pVIII\textsuperscript{9}, five copies of minor coat protein (pIII) crucial for binding to F-pili of bacteria at one end; and a mixture of three to five copies of two different minor coat proteins (pVII and pIX) at the other end (Figure 1)\textsuperscript{10}. All of these coat proteins can be used to display diverse foreign proteins or peptides on the surface of a phage for various applications\textsuperscript{2,11,12}.

Phages have several positive aspects for tissue engineering: negligible toxic effects, ability to form assembled suprastructures, and possibility to display growth factors. Current in vivo clinical trials have demonstrated that the use of phages at high doses did not show acute toxicity or immune response\textsuperscript{13-17}, and Rong, J and his colleagues showed that thin sheets of aligned phage could guide the growth direction of mammalian cells without cytotoxic effects\textsuperscript{18}. These results indicate that phages can be good candidates for use as bio-compatible scaffolds in tissue engineering. Biomimetic artificial extra cellular matrix (ECM) is one of the key issues in tissue engineering. Therefore phage-driven suprastructures might be utilized to fabricate ECM-like scaffolds. Previous reports showed that self-assembled phage could be used to form cell matrix structures having influence on cell adhesion, proliferation, and differentiation\textsuperscript{11,13}. Anna Merzlyak et al.\textsuperscript{9} reported that phage assemblies produced by external flow field could be used for monolayer cell culture. Later, Haibao Zhu et al.\textsuperscript{2} described that the proliferation and differentiation of mesenchymal stem cells (MSCs) could be achieved on grooved phage films forming multilayers in aqueous solution through electrostatic interaction with polylysine. Even though a variety of phage-based scaffolds have been studied, most reports have considered monolayer cell culture systems on an immobilized 2D surface. In addition, only negatively charged phage has been used to form assembled phage structures with the aid of positively charged divalent metal ions\textsuperscript{21} or poly-lysine\textsuperscript{3}. However, these phage-based 2D constructions have limitations in controlling the morphologies of assembled structures. In this report, we demonstrate that positively charged phage particles can be fabricated by genetic and chemical modifications of major coat protein p8, and wild type fd phage (wt-phage) and positively charged phage (fd-SSG) can be applied to form assembled phage network structures for potential use as scaffolds in tissue engineering. In addition, we show that the morphology of the phage assemblies can be controlled by changing the mixing ratio of two types of phages.

RESULTS

Fabrication of APTES conjugated phage

In this study, we used a typical electrostatic self-assembly technique to create phage networks. As the first step, two serine residues were fused to the N-terminus of each copy of the major coat protein. The fd phage was modified by the addition of two serine residues to the N-terminus of the major coat protein, forming a fd-SSG phage. The wt-phage retained the original configuration. The fd-SSG phage was then used to form self-assembled phage networks by electrostatic interaction with polylysine. The resulting phage assemblies exhibited a unique morphology, demonstrating the potential of using genetically modified phages for tissue engineering applications.
coat protein p8 of wt-phage by genetic engineering to create the p8SSG-phage construct. Afterwards, we treated the genetically modified phage particles with APTES solution which is a powerful aminosilane coupling agent in order to modify the N-terminal hydroxyl groups of Ser residues (Figure 1). It is well known that positively charged amine groups of APTES molecules can interact with negatively charged amino acids by cross-linking and form Si-O bonds. Treatment with APTES produced reactive amine groups (NH+, pKa = 10.6) on the phage surface which brings a positive charge. The overall positive charge of the amine functionalized phage surface allowed interaction with wt-phage carrying a net negative charge caused by Glu and Asp residues found at the N-terminus of p8.

**Interaction of APTES- with wt-phage**

To investigate the interaction of wt-phage with APTES-phage particles, each phage sample was subjected to size-exclusion chromatography (SEC). As seen in Figure 2A and B, wt- and p8SSG-phage were detected at a retention time of 47.2 and 47.3 min respectively. When p8SSG-phage was treated with APTES, we observed a single peak at a retention time of 46.9 min with a slight shift compared to untreated phage. The peak area was at least 1.5 orders of magnitude smaller than the peak of p8SSG-phage (Figure 2C). The drop in peak area implied that APTES embedded p8SSG-phage interfered with the absorbance at 280 nm. Notably, APTES-treated p8SSG-phage did not form higher oligomer structures in solution. When APTES-phage was mixed with wt-phage, we observed a broader peak with an initial elution time at around 10 min, indicating that the corresponding apparent molecular masses are higher than that of APTES-treated phage or wt-phage (Figure 2D), and there is a broad range of heterogeneous complex formation in the phage mixture.

**Figure 2.** Elution profiles of phage solutions subjected to size-exclusion chromatography (SEC) with Sephacryl S-500 column. Size-exclusion chromatogram of wt-phage (A), p8SSG-phage (B), APTES-phage (C) and wt- & APTES-phage mixture (D). 1 × 10^11 cfu/mL of wt- and APTES-phage were mixed with 1:1 ratio in the sample loading buffer. 50 μL sample was applied to the column with a flow rate of 0.5 mL/min. The arrow indicates the initiation time of oligomerization of phage complex.

**Characterization of phage aggregation by DLS and Fluorescence Microscopy**

We investigated the diameter distribution of phage aggregates by DLS measurements after mixing different ratios of APTES- and wt-phage solutions. The scattering results of wt-, p8SSG- and APTES-phage are presented in Figure 3. The average particle size of p8SSG-phage was determined as 70 nm shown in Figure 3A. However, the mean diameter of APTES-phage was 150 nm, which is notably larger than that of p8SSG-phage (Figure 3B). As shown in Figure 3B and C, the molar ratios of APTES- to wt-phage mixtures were 1:9 and 9:1, respectively. Highly scattering phage assemblies were obtained in both mixtures, but different size distributions of phage aggregates were detected for different mixing ratios. A shifted maximum bar in the scattering intensity indicates the formation of phage complexes.

When APTES-phage was mixed with wt-phage at a ratio of 1:9, a bimodal distribution of phage aggregates was observed. The second peak of population corresponded to the scattered intensity from phage aggregates with an average size of around 650 nm (Figure 3C). The 9:1 mixture showed a wide range of complexes with diameters ranging from 70 to 1,400 nm with a major size of around 350 nm (Figure 3D). We obtained discrete structures with corresponding fluorescence intensities after immunofluorescence labeling of wt-phage and wt-/p8SSG-phage mixture (Figure 3, inset) with anti-p8 antibodies. Figure 3A shows the fluorescence signal detected for single wt-phage molecules and APTES-phage or wt-phage (Figure 2D), and there is a broad range of heterogeneous complex formation in the phage mixture. To further confirm the morphology of phage assembly, different mixing ratios of APTES- and wt-phage solutions (APTES-
Phage was incubated with wt-phage for 16 hr at 25°C) were imaged by semi-confocal microscopy as shown in Figure 4A and B. The mixing ratios of APTES-phage to wt-phage were 1:9 and 9:1, respectively. When APTES-phages were mixed with wt-phages with a ratio of 1:9, phage particles assembled into linear protofilaments associated in bundle-like structures (Figure 4A).
On the other hand, a 9:1 mixing ratio enhanced the phage cross-linking, forming larger, more inter-connected and finer filamentous meshes (Figure 4B). As the incubation time was increased, the formation of cross-linked networks was enhanced. Light microscopy images indicated that as the ratio of APTES-phage to wt-phage increases, the formation of more filamentous phage networks can be induced which is in good agreement with the higher intensity of scattering at a ratio of 9:1 obtained by DLS measurements.

**DISCUSSION**

In this study, wt-phage and APTES-phage were used to obtain filamentous network structures which are potentially useful for further applications in biotechnology. The formation of phage bundles with different methods such as complex formation with polylysine and β-structured phage assembly has recently been reported. Zhu et al. have described the formation of networks triggered by polylysine and negatively charged phage via intermolecular reactions. They concluded that the stretches of overlaid phage on polylysine could facilitate network formation in the shape of linear bundle structures. Hong Su et al. genetically displayed β-structure-forming and HAP-nucleating peptides on p8 of M13 phage for self-assembly and mineralization for bone repair biomaterials. However, they observed only self-assembled bundles limited in microsize, as two different types of phages are capable of forming intramolecular β-sheet structures. In our study, the surface chemistry of the phage was modified by conjugating APTES to genetically modified p8 coat protein. Then positively charged amine groups of APTES-phage particles were allowed to interact with negatively charged surface amino acids of wt-phage to generate complex assemblies such as fibrils and net-like structures (Figure 1). DLS measurements showed an increase in light scattering intensity of filament diameters following the mixing of APTES-phage with wt-phage (Figure 3). Although size distribution graphs of wt-, p8SSG- and APTES-phage showed particles smaller than 200 nm (Figure 3A and B), mixing of APTES-phage with wt-phage particles resulted in structures larger than 400 nm (Figure 3C and B) indicating complex formation between the phage particles. Additionally, DLS data revealed that the average size of phage complexes can be controlled with different mixing ratios of APTES- and wt-phage. Even though the average diameters of each measured phage sample determined by DLS analyses were comparable, the actual size of filamentous phage is different (-1 μm). This can be explained by hydrodynamic effects of light scattering which are insensitive to the filament length, if using absorbance at 633 nm. We demonstrated that APTES-phages were dispersed in solution without any aggregation by gel filtration and fluorescence microscopy analyses. The morphologies of phage networks were shown to be dependent on different mixing ratios of APTES-phage to wt-phage solutions. Elongated fibril-like structures were created by adding APTES-phage to wt-phage at a ratio of 9:1, whereas thicker filamentous bundles were generated with a 1:9 ratio, which are shown in DLS data in Figure 3. The different shapes of phage assembly can be explained by electrostatic interaction based on the different charge densities of cationic amino groups on APTES-phage and anionic carboxylates from three amino acids (p8-AEGDD) of wt-phages. In our suggested model, the driving force for phage assembly lies in predictable electrostatic interaction and recognition, namely, different numbers of counterions seems to be an important parameter in the overall morphology of phage self-assembly. Furthermore, coupling of APTES to phage might make phage entities more rigid, resulting in APTES-phage surrounded by an excess number of wt-phage particles that could interact with other APTES-phages. Therefore, the formation of phage cross-linking can be well-controlled by different mixing ratios of APTES- and wt-phage. In addition to the different mixing ratios of APTES- and wt-phage, incubation time also had an influence on the shape of phage cross-linked network structure. This assembled product can serve as building blocks to form three-dimensional continuous fibrous networks with increased thickness and porosity similar to natural collagen. With this phage cross-linking technique, it is possible to manufacture tunable three-dimensional suprastructures in the shape of either net-like or linear bundle structures by adjusting the process of phage assembly. The controlling of phage networks can be applied to *in vitro* cell culture technologies, as it has recently been reported that the phage assemblies can accelerate osteoblast cell growth and elongation. Taken together, the combination of highly controlled phage self-assemblies can address biological concerns and lead to the development of more suitable organic-inorganic hybrid scaffolds.

**MATERIALS AND METHODS**

**Preparation of bacteriophage constructs**

The p8-SSG (Ser-Ser-Gly)-phage was constructed by modification of the N-terminus of p8 by a series of assembly PCR reactions as described previously. Phage display vector fd-tet was used to introduce two serine residues into the N-terminus of p8. The insert p8-SSG sequence was replaced from the wild type (wt-) fd phage genome bearing p8-Ala-Glu-Gly (p8-AEG) sequence using four oligonucleotide primers as follows: 1. BsrGI-forward primer, 5′-CTGGTCTGTACACCGTGCAAT-3′
2. NotI-reverse primer, 5′-CCCCACAAAGCCGCTT-3′
3. p8-SSG-forward primer, 5′-CGGTCTACATCTAGAAGCCAC-3′
4. p8-SSG-reverse primer, 5′GCTTTTGCCGGATCGTACCA-
GATGAAGCGAAAAGACAGCATCGGAAC-3'

First, by using fd-tet plasmid as a template, the first segment (p8-SSG) was amplified with the forward primer 1 and the reverse primer 4, including a BsrG1 restriction site followed by an insert sequence. Additionally, the second segment complementary to the reverse primer 4 region including the NotI restriction site was amplified by the forward primer 3 and the reverse primer 2 for the insertion. The obtained PCR product was purified from the agarose gel with a spin column (Qiagen, Germany), digested with BsrG1 and NotI restriction enzymes, and cloned into the fd-tet plasmid by overnight ligation at 16°C with T4 ligase (NEB, Germany). The ligated plasmid was transformed into Escherichia coli MC1061 cells by electroporation. Nucleotide sequence of the inserts was confirmed by DNA sequencing (MWG operon, Germany). Escherichia coli K91BluKan strain (K91BK) (kindly provided by Prof. Dr. Georg P Smith, University of Missouri, USA) was used as a host for the amplification of phage particles. Isolation of phages from the host cells was performed by PEG/NaCl precipitation after growing the transformed K91BK cells in LB-broth supplemented with tetracycline (20 μg/mL) and Kanamycin (100 μg/mL) at 37°C with vigorous shaking (260 rpm) overnight. Final phage pellets were dialyzed against 10 mM PBS at pH 7.4 overnight to remove the remaining PEG. The concentration (cfu/mL) of fd phages was determined by phage titration. Phage suspensions were stored at 4°C.

Silanization of p8SSG-phages with APTES
The p8SSG-phage suspension (1 × 10^{12} cfu/mL) was mixed with APTES (3-Aminopropyltriethoxysilane, Sigma, Germany) solution at 99:1 volume ratio and incubated at 25°C for 24 hr. The mixture was then dialyzed against PBS overnight to remove unbound APTES molecules. Silanized p8SSG-phage at a concentration of 1 × 10^{12} cfu/mL were mixed with wt-fd phage at a concentration of 1 × 10^{11} cfu/mL using different mixing ratios (1:9, 1:1 and 9:1). Mixtures were incubated for 16 hr at room temperature.

Size-exclusion chromatography
SEC was applied to analyze the molecular weights and complexity of phage particles. Among the SEC columns, the size exclusion limit of the Sephadryl S-500 (Amersham Biosciences, Germany) is well suited for analysis of filamentous phage particles (approximate molecular weight of 1.7 × 10^8 Da). Therefore, 50 μL of different phage solutions (1 × 10^{11} cfu) were subjected to the SEC column packed with 30 mL of Sephadryl S-500 resin pre-equilibrated with 10 mM PBS with a flow rate of 0.5 mL/min. Absorption values were measured at 280 nm using a UV-detector.

Dynamic Light Scattering (DLS)
Light laser scattering is used to measure diffusion coefficients and mean values of hydrodynamic diameters of nano-sized particles to confirm binding of wt fd phage to APTES conjugated fd phage. We performed DLS measurements with a Zetasizer1000HS/300HS (Malvern Instruments Ltd.) equipped with a 10 mW 633 nm He-Ne laser and a digital autocorrelator. 2 μL of each phage sample (1 × 10^{11} cfu) was applied for each run. All measurements were carried out in triplicate at scattering angles of 90° and at 25°C.

Semi-confocal and bright field microscopy
Wt-phage, p8SSG-phage and their mixtures were imaged with an Olympus IX51 inverted microscope in phase contrast mode with a 100x oil objective. Images were acquired using a CCD camera (Olympus soft imaging system F view). Following an incubation time of 24 hr at 25°C, phage mixtures were obtained by immunofluorescent staining. 20 μL of phage samples at a concentration of 1 × 10^{11} cfu/mL were first incubated in 2% BSA/PBS for 1 hr at 25°C. Biotin conjugated anti-fd phage antibody (1:5,000, Sigma, Germany) was added and incubated for 1 hr at 37°C. After the antibody incubation, phage samples were mixed with Cy3-conjugated Streptavidin solution (1:1,000, Sigma) and incubated at 25°C for 1 hr. Labeled samples were immediately visualized with the olympus IX51 inverted microscope equipped with a WIBA filter set: GFP: Ex-BP 460/90, BS DM 495, Em-BP 515/50.

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REFERENCES


