A Novel Fluorescent Probe: Europium Complex Hybridized T7 Phage

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We report on the creation of a novel fluorescent probe of europium-complex hybridized T7 phage. It was made by filling a ligand-displayed T7 ghost phage with a fluorescent europium complex particle. The structure of the hybridized phage, which contains a fluorescent inorganic core surrounded by a ligand-displayed capsid shell, was confirmed by electron microscope, energy-dispersive X-ray analysis (EDX), bioassays, and fluorescence spectrometer. More importantly, as a benefit of the phage display technology, the hybridized phage has the capability to integrate an affinity reagent against virtually any target molecules. The approach provides an original method to fluorescently "tag" a bioligand and/or to "biofunctionalize" a fluorophore particle. By using other types of materials such as radioactive or magnetic particles to fill the ghost phage, we envision that the hybridized phages represent a new class of fluorescent, magnetic, or radioprobes for imaging and bioassays and could be used both in vitro and in vivo.

Fluorescent probes play important roles in biology, medicine, and biotechnology (*1*, *2*). Conventionally, a fluorescent probe is made by chemically conjugating a fluorophore molecule or a quantum dot to a biomolecule (such as an affinity reagent). Recently, a few unconventional probes have been developed. For instance, researchers at a number of laboratories have explored the use of aequorea-derived fluorescent proteins (AFP) (*3*, *4*). As an alternative to AFP, tetracysteine-biarsenical labeling also has been demonstrated as a very useful probing tool (*5*). In both cases, the "fluorophores" (fluorescent proteins, such as AFP and tetracysteine-biarsenical complex) are fused to the affinity regents by molecular biology methods instead of chemical conjugation.

The concept for a hybridized phage probe is based on the fact that certain tailed phages build their protein shells first and subsequently condense the nucleic acid within them (*6*). As a result, an empty capsid ("ghost phage" or "pseudo phage") from the tailed phage is stable without interior DNA. The architecture of a ghost phage can function as a unique nanocontainer for uniform fabrication of a nanosized functional (fluorescent, magnetic, or radioactive, etc.) particle inside. Consequently, such a hybridized system contains a functional core and a capsid protein shell.

We chose to use a tailed icosahedral T7 bacteriophage for fabricating a hybridized phage. Structurally, T7 phage consists of a capsid shell, a head−tail connector, tail, and tail fibers. The morphogenesis of T7 and its DNA package have been well documented in the literature, thanks to the major contributions by Dr. William Studier (*7*, *8*). It was demonstrated that empty capsid shells of T7 were assembled prior to the DNA packaging and can be isolated at the early stage of the lytic infection. Given the diameter of T7 phage (approximately 55 nm); a ghost T7 phage provides a cavity of ~40 nm, which can be used to accommodate fluorescent materials, thus leading to a fluorescent material hybridized T7 phage.

An intrinsic advantage of using fluorescent material hybridized T7 phage as a fluorescence probe is that it has the capability to carry a specific affinity reagent against virtually any target molecules via phage display technology (*9*−*11*). In phage display, ligands (such as recombinant antibody fragments, cDNA-encoded segments, or combinatorial peptides chains) are expressed as fusions to a capsid protein present on the surface of viral particles. Libraries of millions to billions of phage particles, each displaying a different fusion protein, are screened (usually by affinity selection) for members displaying the desired properties or binding affinities. Normally, three rounds of screening (biopanning) processes will yield the isolation of a specific binding phage against the target molecule. Subsequently, one can make the corresponding ghost phage and use the ghost as the container to host the fluorescence material inside the ghost to form a hybridized phage. The approach of constructing a fluorescent core and ligand displayed capsid shell structure provides a revolutionary way to "tag" a fluorescence material with affinity reagents or to "functionalize" a bioligand with fluorescence. Thus, the hybridized phage represents a novel class of fluorescent probe.

Among the fluorophores used in the fluorescent probes, the lanthanide complex (such as europium or terbium complex) exhibits unique spectral characteristics (*12*). Lanthanide complexes are superior to conventional organic fluorophores because they have very sharp emission spectra with more than a 300-nm Stoke shift, plus an extremely long fluorescence lifetime (~1 ms), which can be easily detected by time-resolved fluorescence spectroscopy. Thus, lanthanide complexes are the ideal fluorophores to overcome the problems associated with background fluorescence in a biological sample. However, making a lanthanide probe, like making other fluorescence probes, involves chemically conjugating a ligand

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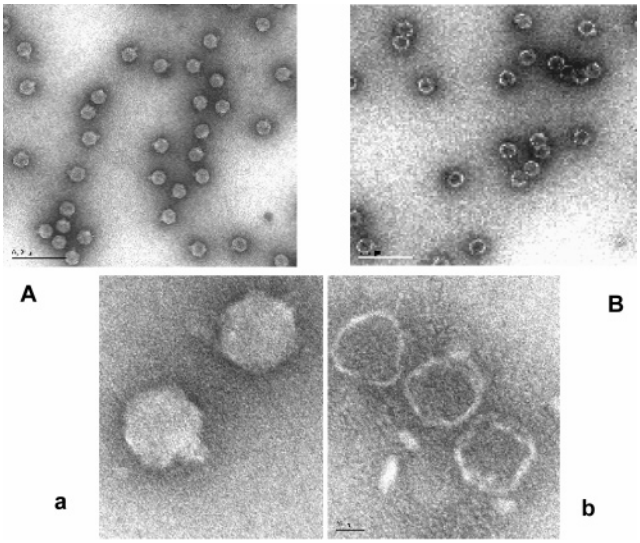

Figure 1. TEM images of uranyl acetate negatively stained T7 phage particles. Left: normal phage particles. Right: ghost phage particles obtained by osmotic shock. Scale bars: 200 nm (a,b: 20 nm).

molecule (affinity reagent) to the chelator molecule, which requires cumbersome synthetic efforts.

In this study, we used a special T7 phage, on which 415 copies of a 15-mer peptide (termed as S-tag) were displayed on its capsid (Novagen). The interaction of S-tag with a 104 amino acid (aa) S-protein derived from pancreatic ribonuclease A has well been characterized in the literature (*13*). After producing the corresponding T7 ghost phage, we were able to synthesize a europium-complex particle inside the ghost phage. Given the unique photophysical properties of europium complex, the hybridized phage can be followed by using both conventional and time-resolved fluorescence spectrometers. Most importantly, the fluorescence hybridized phage retains the binding affinity to the target protein.

T7 ghost was made by osmotic shocking the normal T7 phage. Since the density of encapsulated T7 DNA (~450 mg/mL) is at least 5-fold higher than that in metaphase chromatin (*14*), the DNA of T7 will burst to the outside after the T7 capsid is disrupted by the osmotic shock. By rapidly diluting pure T7 phages (10^{12} pfu/mL) with a sodium sulfate solution (3 M) in the presence of DNAase, it was found that the osmotic shock caused the escape of DNA debris (as a result of DNAase activity) from the capsid shell. However, the integrity of capsid shell remained after removing the osmotic shocking conditions. The shocked phage particles were collected by ultracentrifuging (60 000 rpm for 1 h at 4 °C). The ghost phages were then separated from the normal virus particles by banding in a cesium chloride density gradient (42% CsCl banding position for normal phage and 20.8% CsCl band position for the ghost phage), followed by dialyzing against the PBS buffer solution to remove cesium chloride. Capillary zone electrophoresis also confirmed the generation of T7 ghost phage. On the basis of the peak areas of the ghost particle and survived phage, it was estimated the yield of ghost phages generated by osmotic shock was 55%.

To visualize the ghost particles, phage samples prepared from the above methods were negatively stained with uranyl acetate (1%) and examined by transmission electron microscope (TEM, Philips CM-120). As shown in Figure 1 (A and a), the packing of DNA inside a normal T7 phage is very tight, thus preventing uranyl acetate presented at the virus core of intact viruses, as shown by the brighter contrast on the image. On the contrary, the ghost T7 particles are slightly shrunk, with an average diameter of 48 nm. Since uranyl acetate can diffuse inside the capsid, ghost particles have darker contrast in the middle (Figure 1, B and b). Most of the ghost particles observed did not possess any tails; thus, we assumed that the tails became detached from the particles during the osmotic shock.

Europium-complex hybridized phage particles were synthesized by the reaction of europium ions with either naphthoyltrifluoroacetone (NTA) or dicarboxylic anthraquinone (DCAQ) in the presence of ghost T7 phage (*15*). In brief, ghost T7 particles (0.4 mg/mL) were incubated with 4 mM of europium ions in an acetate acid buffer solution (pH = 8.0) for 1 h. 4 mM of NTA or DCAQ in the same in acetate acid buffer solution was then introduced, and the resulting solution was incubated for 3 h. During the incubation, insoluble europium−NTA or europium−DCAQ complex particles were formed. As the size of the particles increased, the diffusion of europium-complex particles inside the ghost phage were limited by the permeable size of capsid shell; thus, larger europium-complex particles were stuck inside the ghost phage and continued to grow until the particles occupied the entire interior space of the ghost. The formed hybridized phage was purified by using magnetic beads coated with S-protein. Because of the specific interaction between the S-tag displayed T7 ghost and S-protein, the hybridized phages were immobilized on the surface of the beads. Europium-complex particles formed outside the phage were washed away with the acetate acid buffer solution containing 4 mM of NTA or DCAQ solution using a magnetic separator. Finally the hybridized phage was released from the beads using a competing assay by introducing T7 tag elution buffer (T7 Tag affinity purification Kit, Novagen).

The formed europium complex hybridized phage particles have the typical Eu(III) complex luminescence feature. The emission peak is centered at 617 nm when excited at 370 nm, which can be attributed to the $^5D_0 \rightarrow {}^7F_2$ transition (Figure 2A). Thus, the specific binding function of the hybridized phage against S-protein can be verified by a time-resolved fluorescence immunosorbent assay. An intact T7 phage, which went through the same procedures as making a europium complex hybridized phage, was used as a control. Both types of phages (hybridized and intact) were added to the microtiter plate wells coated with the S-protein and incubated at room temperature for 2 h. After the wells were washed three times with TBS, the fluorescence signals of the plate were recorded by a plate reader (Wallac 1420 Victor multiabel counter) equipped with a time-resolved fluorescence detector. As shown in Figure 2B, we observed a strong time-resolved fluorescence signal in the sample of hybridized phage, indicating the strong binding between the S-protein and the hybridized phage (blue line). On the other hand, intact T7 phage only showed very weak signal because of the lack of europium complex (red line). At the same time, a conventional ELISA, using 50 μL of S-protein labeled with horseradish peroxidase (HRP) and ABTS solution for visualization, was also conduced, and the ELISA confirmed the binding of both samples to the S-protein (data not shown here).

To visualize the hybridized phage particles, the samples were loaded onto a freshly glow discharged TEM grid and imaged directly without negatively staining. As demonstrated in Figure 3A, hybridized T7 phages with uniform europium particles inside were dominated on the TEM

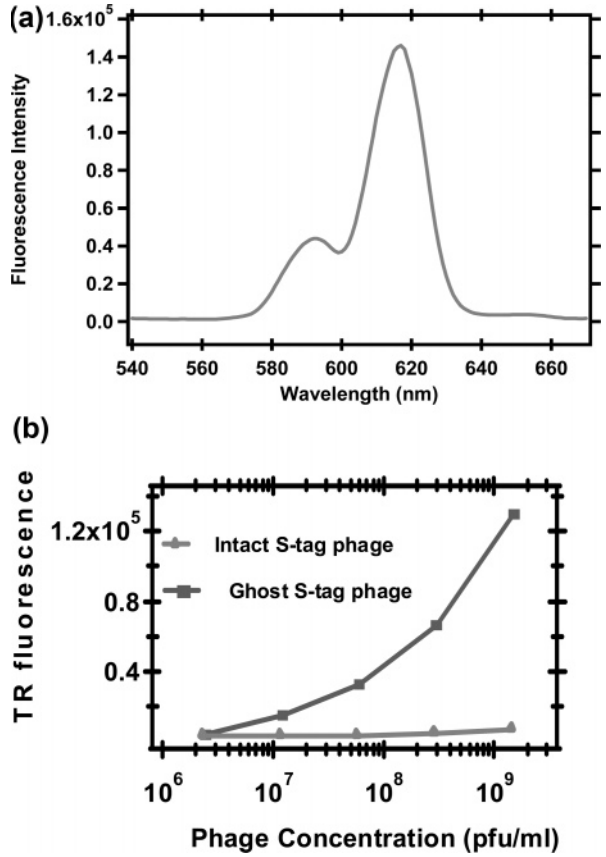

Figure 2. (A) Fluorescence spectrum of europium complex hybridized T7 phage particles. (B) Time-resolved fluorescence immunosorbent assay.

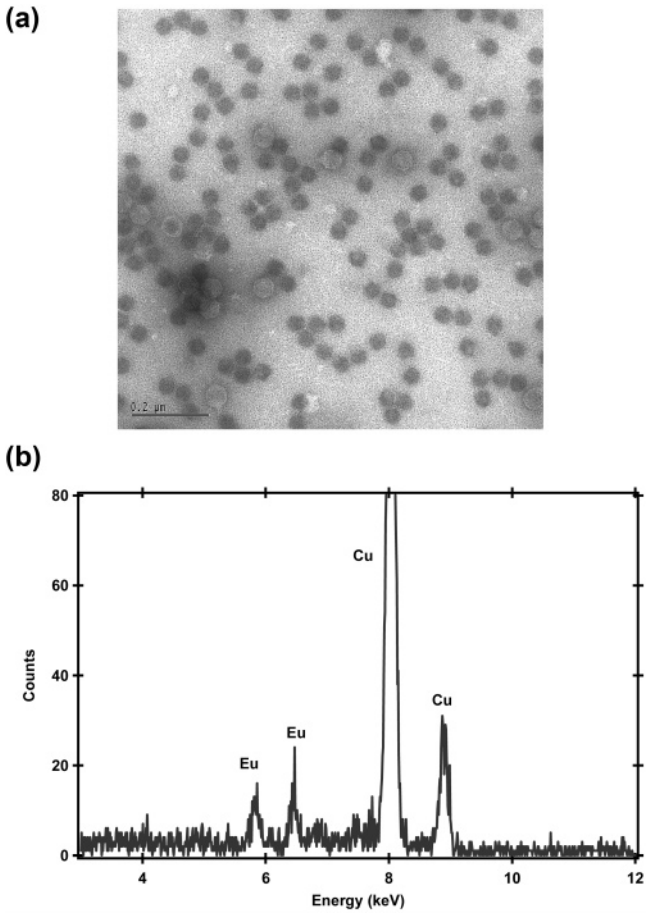

Figure 3. (A) TEM image of europium-complex hybridized T7 phages (scale bar = 200 nm). (B) EDX spectrum indicating the dominated europium element inside T7 ghosts.

images. The size of europium-complex particles inside the ghost T7 is about ~35 nm in diameter with excellent monodispersed size distribution. EDX analysis confirmed the presence of europium (Figure 3B) as the major element inside the ghost T7.

Since the fabrication of europium-complex hybridized T7 involves loading the cavity of T7 ghost with europium ions, a negatively charged phage capsid will help to attract Eu^{3+} ions, thus facilitate the synthesis of europium-complex particles inside the T7 ghost. Although it is hard to describe the exact interior charge nature of the T7 ghost, we estimated that T7 capsid is negatively charged when it is in the solution of neutral pH (~7) or above. This is supported by the fact that the capsid of T7 is made of many (up to eight) capsid proteins. The isoelectric point (pI) of major capsid protein (P10) is 6.0, and the pIs of dominated internal protein (P15 and P16) of T7 capsid are 5.4 and 6.6, respectively (*16*). The negatively charged nature of the T7 ghost phage at neutral or base conditions benefits the loading of cationic europium ions, and so europium complex particles can be efficiently synthesized inside the T7 ghost.

It should be noted that the observed time-resolved fluorescence signal is almost 3 orders of magnitude higher than the background noise and nonspecific signals in the time-resolved fluorescence immunosorbent assay experiments. Compared to a conventional ELISA assay, the europium-complex hybridized phage provides a superior signal-to-noise ratio as a fluorescent probe.

In summary, we have demonstrated the generation of a europium-complex hybridized T7 phage. As a new class of fluorescent probe, hybridized phages have monodispersed europium-complex particle core inside and uniform affinity reagent displayed capsid shell outside. Currently, we are expanding the scope of hybridized phage and have been successful in synthesizing cobalt metal and rhenium oxide (an analogue to ^{99}technetium oxide due to similar chemical properties of these two elements) hybridized phages. Since the inorganic cores in the hybridized phages are fully surrounded by the capsid proteins, in principle, the hybridized phage should be biocompatible in vivo. We envision that the hybridized phage will be the next generation of probing reagents for bioassay, biosensor, targeted reagent delivery, and medical imaging.

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