Phage-Derived Bioselective Nanovehicles For Drug And Gene Delivery

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ABSTRACT

Multibillion-clone landscape phage display libraries were prepared by the fusion of the major coat protein pVIII with foreign random peptides. Phage particles and their proteins specific for cancer cells were selected from the libraries and converted into the targeting interfaces of the gene- and drug-delivery systems by encapsulation of the foreign phagemid DNA by phage coat proteins *in vivo* and by incorporation of the cell-specific phage fusion proteins into liposomes *in vitro*. As paradigms, targeted pVIII proteins were exploited for encapsulation of the marker green fluorescent protein (GFP) gene and for incorporation into the therapeutic liposomes "Doxil". Ability of the gene- and drug-encapsulating nanoparticles to bind target receptors was evidenced by fluorescent microscopy, fluorescence-activated cell sorting, microarray, optical and electron microscopy. In contrast to a poorly controllable conjugation targeting, the new landscape phage-based approach relies on very powerful and extremely precise mechanisms of selection, biosynthesis and self assembly, in which phages themselves serve as a source of the final product.

Keywords: drug and gene delivery, targeting, phage display, landscape library, phagemid, liposome, nanoparticle.

1 INTRODUCTION

Filamentous phages f1, fd and M13 are nonlytic bacterial viruses, which structure and life cycle are encrypted in their single-stranded DNA genomes **(Fig. 1)**. The whole architecture and function of the phage rode-like particles can be manipulated using rational and combinatorial engineering of their genes encoding five coat proteins—the major coat protein pVIII, forming the phage capsid and consisting 98% of the total protein mass, and the five minor coat proteins located at the capsid's edges [1, 2]. The combinatorial manipulation of the viral genes resulted in design of *phage display libraries*—multibillion clone compositions of self-amplified and self-assembled biological particles, displaying foreign peptides on their surfaces [6]. In particular, a paradigm of *landscape phage libraries* evolved, in which the phage is considered as a nanoparticle (nanotube) with emergent physico-chemical characteristics determined by specific phage landscapes formed by thousands of random peptides fused to the major coat protein pVIII [8].

There is a fast growing interest to the landscape phages as a new type of selectable nanomaterials (reviewed in [2]). The landscape phage can bind organic ligands, proteins and antibodies, induce specific immune responses in animals, or resist stress factors such as chloroform or high temperature. Landscape phages have been shown to serve as substitutes for antibodies against cellular antigens and receptors, diagnostic probes for bacteria and spores, and biospecific adsorbents. Phage-derived probes inherit the robustness of the wild-type phage and allow fabrication of bioselective materials by self-assemblage of phage or its composites on metal, mineral or plastic surfaces. Landscape phages specific for bacterial cells and spores have been exploited as recognition interfaces in detection systems [11].

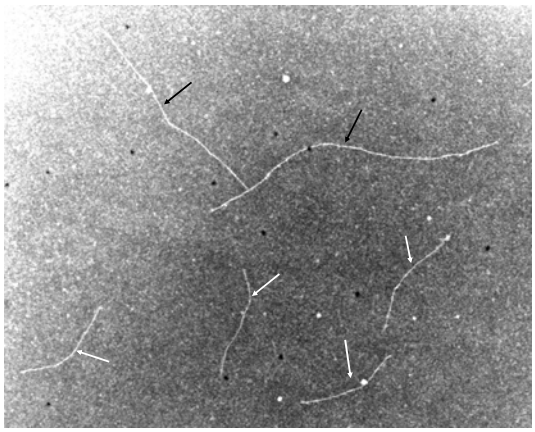

Fig.1. Transmission electron micrograph of phage (pointed out by black arrows) and targeted phagemid infective particles PIP (white arrows). Original magnification 57,000X. Adapted from [6].

Here we outline the use of the landscape phage as a bioselective interface in gene and drug delivery systems. The concept of using targeted pharmaceutical nanocarriers to enhance the efficiency of anti-cancer drugs has been proven over the past decade both in pharmaceutical research and clinical setting. In particular, it is commonly accepted that selectivity of drug delivery systems can be increased by their coupling with peptide and protein ligands targeted to differentially expressed receptors. The abundance of these receptors was demonstrated recently by comparative analysis of gene expression in tumor cells and tumor vascular endothelial cells versus adjacent normal tissues [7], and their targeting is turning into a routine procedure in the most advanced laboratories due to the progress in combinatorial chemistry and phage display (a long list of different target-specific peptides identified by phage display can be found in recent reviews [1,12]. In particular, selection protocols were developed for obtaining the phages and peptides internalizing into cancer cells, and

phage homing at tumors of human patients. Selection of tumor-binding phage ligands in cancer patients, demonstrated recently by David Krag with colleagues, opened a new avenue for applications of phage technology for patient-directed cancer treatment [8].

A new challenge, within the frame of this concept, is to develop highly selective, stable, active and physiologically acceptable ligands that would navigate the encapsulated drugs to the site of the disease and control unloading of their toxic cargo inside the cancer cells. We have shown earlier that the tumor-specific peptides fused to the major coat protein pVIII can be affinity selected from multibillion clone landscape phage libraries by their ability to bind very specifically cancer cells [16, 19]. Here we show that the target-specific phage can be converted easily into the gene-encupsulating particles, or drug-loaded vesicles. Thus, the major principle of our approach is that targeted therapeutic nanoparticles recognize the same receptors, cells, tissues and organs that have been used for selection of the precisely targeted landscape phage.

2 TARGETED GENE DELIVERY BY CANCER CELL-SELECTIVE PHAGE PROTEINS

A fundamental requirement for a cancer therapeutic gene delivery device is that it has the ability to enter into a tumor compartment, target cancer cells leaving surrounding tissue unscathed, and deliver into the cancer cells a therapeutic gene that will stop their growth and cure the disease. Different structural elements of the device may be dedicated to various steps of this process. The therapeutic gene can be expressed in the cancer cell constitutively or may be activated specifically by a cancer cell product. Thus, to optimize the cancer treatment strategy, a system is required which would be versatile enough to allow using both selection and rational design for its development.

To meet these requirements, we developed a gene delivery system**,** which is based on phagemid infective particles (PIPs) encapsulated within bacteria by peptides encoded by a tumor-selected landscape phage [6]. These particles are considerably smaller than normal phage (~700 nm *vs*. ~1300 nm; Fig. 1), but have all the elements necessary for delivery and expression of genes in mammalian cells. Their DNA can harbor a therapeutic gene, mammalian and bacterial origins of replication, as well as antibiotic resistance genes, used as markers. All the proteins required for encapsulation of the phagemid DNA, including tumor-targeting peptides, are provided by a phage helper. The strategy was justified by using a helper phage that is internalized by RG2 glioma cells selected from a landscape phage library [9]. It was shown that the phagemid infective particles, PIPs are able to bind and penetrate into the target cancer cells and express the marker gene from within the cell. Antibody test showed that about 85% of the particles were internalized by the cancer cells **(Fig. 2).** However, the low expression level of the marker GFP gene (2-3%) probably indicates a breakdown in the trafficking/expression pathway. This is not surprising since little effort was made to enhance PIPs DNA escape from the endosome.. Transformation of eukaryotic cells with single stranded DNA phages can be increased if they are supplied with inverted terminal repeats of adeno-associated virus (AAV) [12].

The approach, shown for glioma cells, differs from

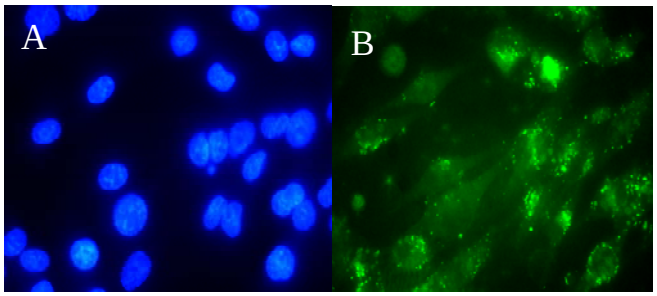

Fig. 2. RG2 cells treated with PIPs. A: DAPI florescence associated with each individual cell's nuclears; B: the fluorescence of the FITC-conjugated goat anti-rabbit antibody associated with the internalized PIPs particles. Adapted from [6].

others in that a phagemid expressing a model marker or particular therapeutic gene can be easily exchanged for a phagemid expressing a different therapeutic gene. Also, a different helper phage, selected from a phage display library can target any cell type and direct the encapsulation of any therapeutic gene encoding phagemid. Because of its versatility, the PIP system may be readily used for optimization of the gene-delivery strategies applied to specific cell and tissue targets.

3 DRUG TARGETING WITH THE STRIPPED LANDSCAPE PHAGE

The ability of the major coat protein pVIII to form micelles and liposomes emerges from its intrinsic function as membrane protein judged by its biological, chemical, and structural properties. During infection of the host *Escherichia coli*, the phage coat is dissolved in the bacterial cytoplasmic membrane, while viral DNA enters the cytoplasm [4]. The protein is synthesized in infected cell as a water-soluble precursor, which contains a leader sequence of 23 residues at its N-terminus. When this protein is inserted into the membrane, the leader sequence is cleaved off by a leader peptidase. Later, during the phage assembly, the processed pVIII proteins are transferred from the membrane into a coat of the emerging phage. Thus, the major coat protein can change its conformation to accommodate to various distinctly different forms of the phage and its precursors: phage filament, intermediate particles and membrane-bound form. This structural flexibility of the major coat protein is determined by its unique architecture, which is studied in much detail [5]. In virions, mostly α-helical domains of pVIII are arranged in

layers with a 5-fold rotational symmetry and approximate 2-fold screw symmetry around the filament axis. In opposite, in the membrane-bound form of fd coat protein, the 16-Å-long amphipathic helix (residues 8-18) rests on the membrane surface, while the 35-Å-long trans-membrane (TM) helix (residues 21-45) crosses the membrane at an angle of 26° up to residue Lys40, where the helix tilt changes. The helix tilt accommodates the thickness of the phospholipid bilayer, which is 31 Å for *E. coli* membrane components. Tyr 21 and Phe 45 at the lipid–water interfaces delimit the TM helix, while a half of N-terminal and the last C-terminal amino acids, including the charged lysine side chains, emerge from the membrane interior. The transmembrane and amphipathic helices are connected by a short turn (Thr 19–Glu 20).

3.1. Stripped Phage Proteins

In model experiments, landscape phages were converted to a new biorecognition affinity reagent—"stripped phage" [3]. The stripped phage is a composition of disassembled phage coat proteins with dominated (98%) recombinant major coat protein pVIII, which is genetically fused to the foreign target-binding peptides. The stripped phage proteins can form bioselective vesicles decorated by target-binding peptides, which can be used for the targeted drug delivery (Fig. 3). In our example, the stripped phages were prepared

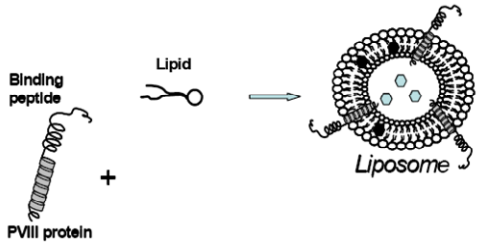

Fig. 3. Drug-loaded liposome targeted by the pVIII protein. The pVIII spans the lipid bilayer and displays the binding peptide on the surface of the carrier particles. The drug molecules are shown as hexagons

by treatment of the landscape phages with chloroform followed by conversion of resulted spheroids into the lipid vesicles by their reconstruction with phospholipids. In preliminary experiments with streptavidin- and bacterial binders it was demonstrated by competition ELISA, acoustic wave sensor and transmission electron microscopy that the stripped phage proteins retain the target-binding properties of the selected phage [3].

3.2. Fusion of the Stripped Phage Proteins with Doxil

Using intrinsic mechanism of fusion of the phage proteins with lipid membranes, we incorporated streptavidin-targeted proteins into the commercially available Doxil liposomes. The streptavidin-binding landscape phage was affinity selected from 9-mer landscape library. The phage was converted into spheroids with chloroform and incubated with Doxil to allow fusion of the phage proteins with liposome membrane, as illustrated by Fig. 3. As a result of the phage fusion, the liposome acquired a new emergent property—ability to bind

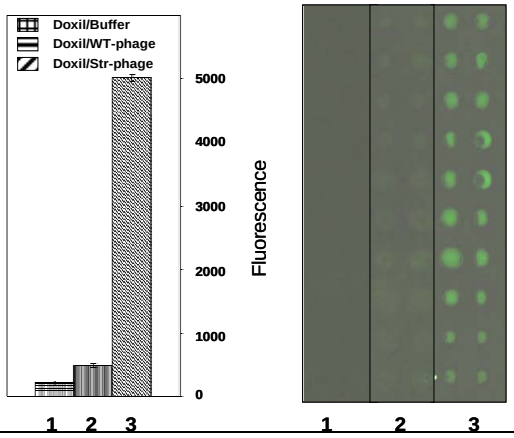

Fig. 4. Microarray test for Doxil targeting. Streptavidin-microarrayed slide was treated with unmodified Doxil (1), Doxil modified with wild-type phage (2) and Doxil targeted with streptavidin binding phage (3).

streptavidin and streptavidin-conjugated fluorescent molecules, as was evidenced by protein microarrays, fluorescent microscopy and fluorescence-activated cell sorting (FACS). The targeted and control liposomes were incubated with streptavidin-coated chips, washed and scanned (Fig. 4), or mixed with Texas Red-conjugated streptavidin (TRS), washed and analyzed by fluorescent microscopy and FACS. Complex of the modified Doxil with the target streptavidin demonstrated 50-fold higher fluorescence than pure Doxil and 10-fold higher fluorescence than control Doxil. The complex of the targeted Doxil liposomes with streptavidin-coated gold beads was visualized by transmission electron microscopy (Fig. 5).

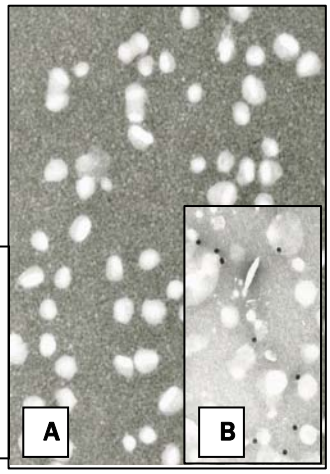

Fig. 5. Doxil liposomes (A), and complexes of the targeted Doxil liposomes with streptavidin-coated 20 nm gold beads. An evarage size of unmodified Doxil particles – 80 nm.

4. CONCLUSION

We proved the novel approach to the specific targeting of therapeutic/imaging genes or pharmaceuticals through their enclosing into the capsules formed by the stripped fusion proteins of the affinity selected landscape phage. The phage specific for the target organ, tissue or cell is selected from the multibillion landscape phage libraries, and is combined with foreign DNA or liposome-entrapped drugs exploring intrinsic self assemblage properties of the phage proteins. As a result, the targeting probe—the target-specific peptide fused to the major coat protein—is exposed on the shell of the gene- or drug-loaded vesicle. In contrast to sophisticated and poorly controllable conjugation procedures used for coupling of synthetic peptides and antibodies to the targeted vesicles, the new phage-based concept relies on very powerful and extremely precise mechanisms of *selection, biosynthesis and self assembly*. When landscape phage serve as a reservoir of the targeted membrane proteins, one of the most troublesome steps of the conjugation technology is bypassed. Furthermore, it does not require idiosyncratic reactions with any new shell-decorating polymer or targeting ligand and may be easily adapted to a new liposome or micelle composition and a new addressed target. No reengineering of the selected phage is required at all: the phages themselves serve as the source of the final product—coat protein genetically fused to the targeting peptide. A culture of phage-secreting cells is an efficient, convenient and discontinuous protein production system. They are secreted from the cell nearly free of intracellular components; their further purification could be easily accomplished by simple steps that do not differ from one clone to another. The major coat protein constitutes 98% of the total protein mass of the virion — the purity hardly reachable in normal synthetic and bioengineering procedures. As a normal intestinal parasite, phage itself and its components are not toxic and have been already tested for safety in preclinical and clinical trials [8,10]. In contrast to immunization procedure, the phage selection protocol does not meet the tolerance problem, which can hamper obtaining antibodies to "self" antigens, and may require tiny amounts of a target material (thousands of tumor cells available in biopsy procedure [8] for obtaining the tumor-specific phage ligands, affinity and selectivity of which may be controlled by exploring well developed depletion and affinity maturation procedures.

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