Self-navigating drug delivery nanovehicles driven by polyvalent multifunctional phages and their promiscuous proteins

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ABSTRACT

In the early 1900s Paul Ehrlich pioneered the concept of a therapeutic 'magic bullet' and ushered in the era of modern 'precision medicine' research. The idea that a molecular probe could be delivered specifically to targeted cells within the organism, was the 'Holy Grail' for several generations of scientists. With the development of *nanomedicines*, significant progress towards targeted cancer chemotherapy have been achieved, mostly through exploring passive targeting based on Enhanced Permeability and Retention (EPR) effect. Discovery of unique, organ vasculature-specific receptors, '*zip codes*', opened a new avenue for organ- and tumor-directed drug delivery [3]. The active targeting of nanomedicines towards the vasculature and tumor cells has been proposed to enhance their therapeutic efficacy [4]. However, the first attempts of adapting the concepts of direct tumour targeting has shown minimal improvements [5]. Analysis of nanoparticle delivery data accumulated during the past decade demonstrated that only a tiny portion of the administered nanoparticle dose is delivered to solid tumors, that creates a critical hurdle for translating nanomedicines into the clinic [6, 7]. Furthermore, based on numerous clinical studies it was concluded that the EPR effect—the basic rationale of the design and development of nanomedicines in cancer therapy—works in rodents but not in humans [8]. The low performance of targeted nanomedicines in biological experiments forced researchers to pursue other ways of exploiting nanomedicine's pharmaceutical potential in cancer therapy, even if the fundamental concepts of EPR effect and tumor targeting are compromised. Unfolding the complexity of the tumor microenvironment has revealed biological barriers hindering efficacy of the targeted drug delivery in human patients. To overcome these barriers, it was suggested to supply the tumor-targeting vehicles with *multiple ligands* targeted to different components of the tumor environment. Following this trend in using '*molecular cocktails*' for cancer drug targeting, which will likely prevail in the development of advanced targeted nanomedicines, we suggested a novel '*addressed self-navigating drug*' concept, in which ligands selected using traditional principles of affinity [9] are substituted for ligands developed through '*migration selection*' — multifunctional phage particles able to

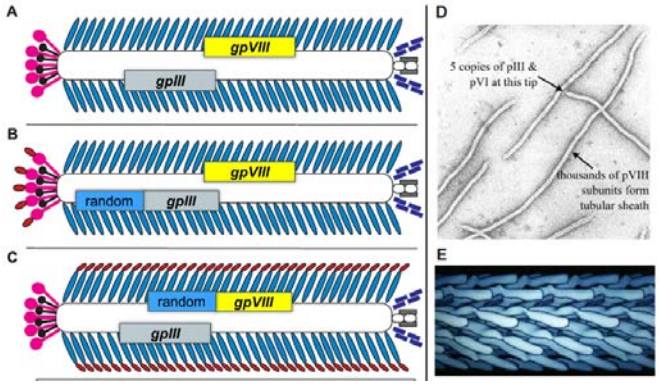

Fig.1. Phage display. A: phage vector composed of 4,000 copies of the p8 major coat protein **(blue)** encapsulating a ssDNA, and minor coat proteins pIII **(pink)**, pVI **(black)**, pIX (**gray**), and pVII **(purple)**. **B, C:** p3 and p8 phage display libraries. A random peptide **(red)** is fused to every copy of either p3 or p8 proteins. **D:** TEM of bacteriophage fd. **E:** Electron density model of the major coat protein p8 in the fd phage.

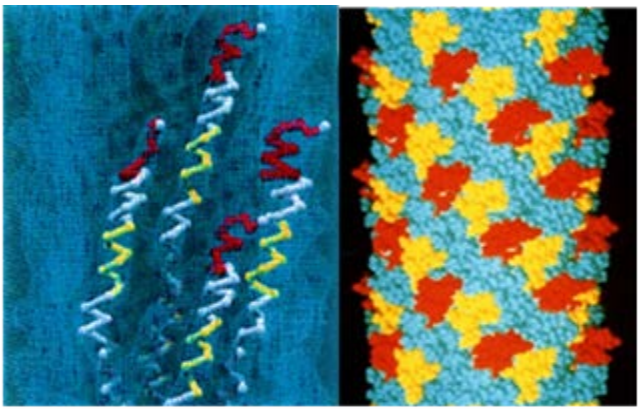

Fig.2. Models of landscape phage libraries. Left: Ball-and-stick model of four neighbor p8-proteins with inserted N-terminal random foreign peptides (**red**) and mutagenized central segments (**yellow**) on the surface of the phage capsid (**blue contour**). About 1% of the phage length is shown. **Right:** Random foreign peptides grafted into the major coat protein. N-terminal foreign peptides are pictured with red atoms, central domains are pictured with yellow atoms; their specific structural and positional details are entirely speculative but their overall arrangement is presumably accurate. The distance between neighboring peptides is ~ 2.7 nm.

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extravasate from the blood stream to the tumor tissue, migrate through the molecular/cellular barriers surrounding tumors, penetrate into the tumor mass and attack the diverse tumor cell population [10]. We hypothesized that peptide motifs, discovered through *migration selection* would serve as '*elementary binding units*' and can be used as '*molecular LEGO*' for construction of proteins with expected tissue migrating propensity.

Keywords: cancer; nanomedicines; drug delivery; gene delivery; landscape phage; nanobiotechnology; phage display; phage proteins

1. DEVELOPMENT OF PHAGE PROTEIN-TARGETED NANOMEDICINES

In our preliminary experiments, we used our proprietary landscape phage display libraries f8/8 and f8/9 **(Fig.1,2)** to select clones with specificity to various cancer types [1, 11-20] and demonstrated that phage fusion proteins selectivity interacting with various cellular phenotypes are ideal construction material for preparation of targeted nanomedicine platforms (**Fig.4**) [1]. In collaboration with Dr. Torchilin's group, we first proved the use of the phage coat proteins for targeting of drug-loaded liposomes and micelles to cancer cells, increasing their anti-tumor potential towards human breast, prostate, pancreatic and lung cancer cells [2, 21, 22]. The phages binding and penetrating into the target cancer cells were selected from landscape phage

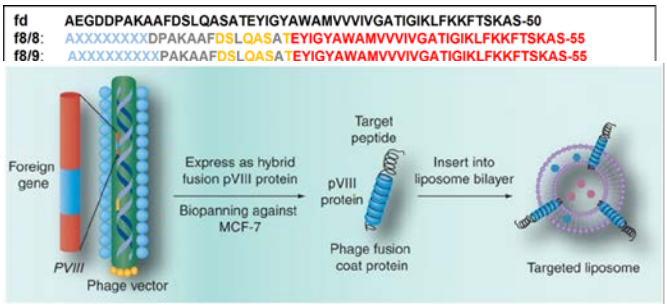

Fig. 3. Top: Major coat protein fd and its fusions with 8-mer and 9-mer random peptides in "landscape" phage display libraries; X – random amino acid. **Bottom**: Production of phage coat protien fused with tumor targeting peptide and its incorporation into the liposome bilayer.

libraries and their fusion coat proteins were inserted into the liposomes and micelles exploring their intrinsic membrane properties, as shown in **Fig.3**. It was shown that the specificity of selected phages and their proteins towards cancer cells translates to the protein-modified nanomedicines, increasing their specific binding and cytotoxic activity towards the target cancer cells. *The major principle of our approach is that targeted nanomedicines recognize the same receptors on the target cells, which have been used for selection of the precisely targeted landscape phages.* The procedure developed in our study circumvents the complex, protracted and poorly controllable conjugation procedures used for coupling synthetic peptides and

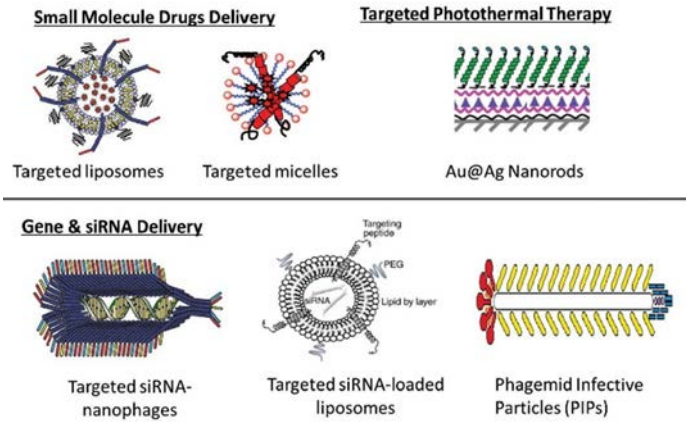

Fig.4. Nanomedicine platforms derived from fusion phage proteins [1]

antibodies with nanovesicles and instead uses the extremely precise natural mechanisms of selection, biosynthesis and self-assembly of filamentous phages.

2. DEVELOPMENT OF DUAL-TARGETED NANOMEDICINES

The major hurdles that hamper efficient clinical application of tumor targeted nanomedicines are physiological barriers that they have to overcome after leaving the leaky tumor vasculature and before they can bind and kill cancer cells. For example, a drug delivery vehicle able to cross the blood-brain barrier while potentially targeting a specif ic group of cells should be able to find the CNS, cross the BBB without harming its integrity, find and attack cancer cells inside the brain and release the therapeutic agent in the proper compartment of the targeted cell [10]. To respond to these concerns, we focused on development of ***dual (multiple) targeted (DT) nanomedicines***, which are driven by two or more different ligands accommodated on the same nanoparticle, for example one ligand targeted against tumor cells and another - against the tumor vasculature [23, 24] **(Fig.5, right)**.

The fusion major coat proteins demonstrated excellent performance in design of dual targeted nanomedicines, both as a construction material and as a specifically targeted

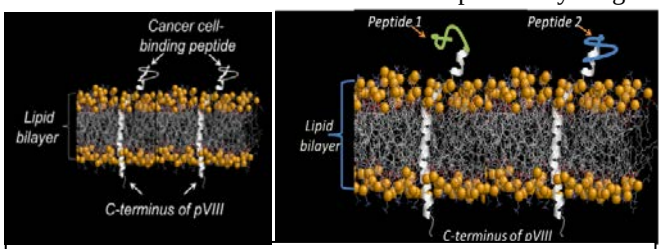

Fig. 5. Models of mono- **(left)** and dual targeted **(right)** nanomedicines showing lipid bilayer along with one (left) or two different inserted proteins (right)

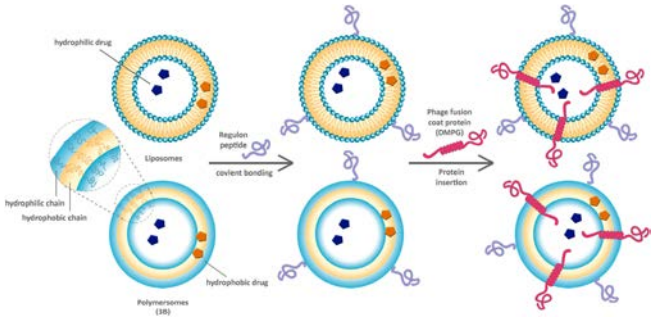

Fig.6. Schematic representation of dual-targeted liposomes and polymersomes [2].

ligands. For example, dual-targeted liposomes and polysomes were constructed to treat metastatic breast cancer in the brain, combining the use of previously selected MCF-7-specific phage fusion pVIII coat protein and a 59-residue peptide of LRP-1 (Regulon Inc.), used as a model BBB penetrating moiety (**Fig.6**) [2].

3. SELF-NAVIGATING DRUG DELIVERY VEHICLES

To respond to the evolving concern about using targeted nanomedicines for *in vivo* drug delivery—suboptimal EPR effect in humans and slow penetration into the tumor microenvironment [6, 8]—we focused on development of ***targeted self-navigating nanomedicines***, driven by two or more different fusion phage proteins accommodated on the same nanoparticle, as illustrated in **Fig. 5,6.**

Studying homology of hundreds thousands of tumor-binding phage-displayed peptides, we identified short linear *motifs* containing 3-4 amino acid residues, which accumulate in the displayed peptides during different rounds of selection [25], as examplified in **Fig.7**. Discovery of short motifs serving as *elementary binding units* during phage selection inspired us to propose the novel "*addressed drug navigation*" concept, which relies on the use of "*molecular self-navigating ligands*", selected from tissue-migrating polyvalent multi-motif landscape phage display libraries and accumulating '*elementary binding units*' responsible for binding to different tissue cells [26], as illustrated in **Fig.7,8,9**. Applied to the targeted drug delivery problem, this

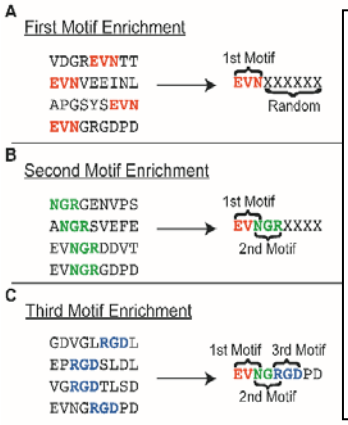

Fig.7. Hypothetical 'motif assembling' mechanism operating during selection of migrating phage. In the 1st round of selection, using lung cancer cells as a target, the **ENV** primary motif was enriched. During subsequent rounds of selection, secondary motifs **NGR** and **RGD** were enriched.

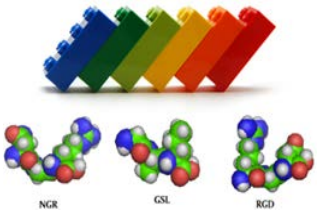

Fig.8. Molecular LEGO: examples of 3-mer peptides—hypothetical 'elementary binding units' discovered during phage selection.

novel approach promises to replace the existing '*point to point*' targeting concept for the novel '*self-navigating*' drug delivery paradigm that can be used as a theoretical basis in development of a novel generation of molecular imaging probes and medications for precise and personal medicine. The novel generation of molecular probes would allow development of advanced self-navigating drugs, imaging probes and nanomedicines able to overcome biological and technical barriers that prevent their precise delivery.

4. PROSPECTS

The advanced bacteriophage-driven self-navigating nanomedicines are thought to overcome biological and technical barriers that prevent tumor drug delivery. Using promiscuous fusion phage proteins and their combinations as self-navigating ligands would allow overwhelming the technically challenging complexity of rationally designed *dual-targeted nanostructures* proposed to increase performance of nanomedicines and extand it to *multiple-targeted nanomedicines*. Taking into consideration the power of molecular selection and simplicity of preparation and assembly of phage proteins into the '*self-navigating nanomedicines*', we can envision a principal breakthrough into a novel era of selective, safe, efficient and economical anticancer medicines that would help to improve the quality of life and save lives of millions cancer patients. Furthermore, the proposed paradigm of '*self-navigating nanomedicines*' can be adapted for development of a novel generation of medications for precise and personal medicine.

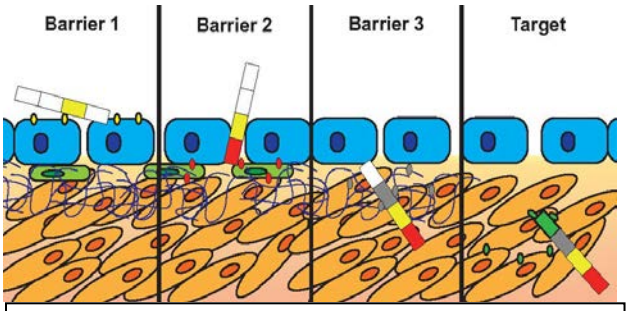

Fig.9. **Selection of mosaic phage proteins that navigate phage through different barriers towards the target cancer cells.** Functional *motif*s accumulate within proteins during phage library migration. The first motif (**yellow**) is selected against tumor endothelial cells. The next *motifs* (**red and grey**) allow penetration through stromal cells and the extracellular matrix. A finally selected *motif* (**green**) is responsible for specific interaction with the target cancer cells and penetration into the correct subcellular compartment.

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