NEURAL REGENERATION RESEARCH
June 2015, Volume 10, Issue 6
www.nrronline.org

HIGHLIGHTS

# Phage display: development of nanocarriers for targeted drug delivery to the brain

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***doi:****10.4103/1673-5374.158330*
***http:****//www.nrronline.org/*

***Accepted:*** *2015-04-28*

## Abstract

The blood brain barrier represents a formidable obstacle for the transport of most systematically administered neurodiagnostics and neurotherapeutics to the brain. Phage display is a high throughput screening strategy that can be used for the construction of nanomaterial peptide libraries. These libraries can be screened for finding brain targeting peptide ligands. Surface functionalization of a variety of nanocarriers with these brain homing peptides is a sophisticated way to develop nanobiotechnology-based drug delivery platforms that are able to cross the blood brain barrier. These efficient drug delivery systems raise our hopes for the diagnosis and treatment of various brain disorders in the future.

***Key Words:*** *blood brain barrier; phage display; peptide library; nanocarrier; targeting*

*Bakhshinejad B, Karimi M, Khalaj-Kondori M (2015) Phage display: development of nanocarriers for targeted drug delivery to the brain. Neural Regen Res 10(6):862-865.*

## Introduction

Drug delivery to the brain remains a hotly debated and hugely studied area in biomedicine. The transport of a vast majority of therapeutic molecules to the brain is restricted by the presence of a natural obstacle called the blood brain barrier (BBB) (Bicker et al., 2014). This limitation highlights the need to create systems idealized for the delivery of therapeutic as well as imaging agents across the BBB, thereby distributing drug molecules to desired site in the brain.

Bacteriophages (phages) as viruses that propagate their genetic material through infecting bacterial cells in a specific manner are thought to be the most ubiquitous biological entities in the biosphere. These viral agents in many ways are superior nanomaterials. High levels of structural organization, self-assembly into structures with nanoscale properties, well-defined geometry, and the flexibility of their small genomes to be conveniently engineered have adapted them for the fabrication of nanostructured materials. These characteristics have served as a basis for developing phage display technology. Phage display is a high-throughput and combinatorial screening methodology that can be used for building large nanomaterial libraries and subsequently identifying novel ligands with desirable attributes. To date, major part of the work within the field of phage nanobiotechnology has been focused on filamentous bacteriophages in particular M13 and its related phages including f1 and fd. M13 as the most predominantly used type of phage nanoparticles is a flexible and rod-shaped viral nanostructure nearly 6.5 nm in diameter carrying a single stranded DNA genome (Yang et al., 2013). Nanomaterial libraries constructed by using filamentous phages such as M13 are regarded as a treasure of targeting ligands especially cell- and tissue-specific peptides. Therefore, phage display libraries represent huge potential for formulating targeted drug delivery platforms.

Nanocarriers are emerging as attractive tools for the design of brain-targeted drug delivery vehicles. The surface of these nanoscale systems can be decorated with homing peptides derived from phage display libraries. These surface functionalized nanovehicles show optimized selectivity for brain drug delivery. As a result, these platforms may enable delivery of nontransportable drugs across the BBB. In this article, we present an overview of potential application of phage display with a particular focus on phage peptide libraries for the development of brain-targeted drug delivery nanocarriers.

## BBB: a Hurdle for Brain Drug Delivery

The birth of the term BBB stems back more than a century ago and the experiments undertaken by Paul Ehrlich in the late 19$^{th}$ and early 20$^{th}$. He noticed that the direct injection of some dyes locally into the brain could lead to the staining of this organ. However, dye peripheral injection results in the staining of the whole body except the brain (Bradbury, 1979). Specialized cerebral endothelial cells (CECs) with their unique restrictive properties form the major anatomical and functional components of BBB (**Figure 1**). These endothelial cells, known as the brain gatekeepers, constitute the complex and extremely well-organized vascular system of the brain. Adjacent endothelial cells lack intracellular fenestrations and are connected through high resistance tight junctions that turn the brain endothelium into a continuous layered structure keeping the blood apart from the brain. The high structural integrity of tight junctions results from a delicate and intricate network of transmembrane proteins and some cytoplasmic accessory proteins that are linked to the cellular cytoskeleton. Astrocytes, pericytes, microglia, and the extracellular base membrane are other essential parts of the BBB supporting system. These components, together with nearby neurons, create a hugely coordinated system called "neurovascular unit" (Bicker et al., 2014).

A well-coordinated and persistent control over the composition of extracellular fluid in the central nervous system is of particular importance to the efficient function of neurons. In the invertebrates and some ancestral vertebrates, a barrier composed of glial cells safeguards not so complex nervous system of

these organisms from unwanted effects of alterations occurring in the body fluids. However, in the higher vertebrate organisms with an increased complexity in the structure of the nervous system during evolution, an endothelial barrier provides a dramatic protection to the brain. BBB plays a critical role in maintaining homeostasis of the delicate nervous tissue through creating a physical, transport, metabolic and immunological barrier between the blood and the brain. This barrier is highly selectively permeable with the main role of protecting the brain from physiological fluctuations that occur in the plasma as well as from the blood-borne compounds that may interfere with neurotransmission. It also offers a means for specific exchange of essential nutrients, minerals, ions, metabolic waste products, and signaling molecules between the blood and the brain, thereby providing an exquisite and dynamic control to the influx and efflux of different metabolites (Redzic, 2011).

The physiological characteristics of BBB in the selective delivery of various compounds may constitute a challenge for the transport of pharmacologically active molecules to the nervous system. In this context, BBB poses tremendous limitations to the brain-targeted delivery of drugs, while many diseases that affect the central nervous system require therapeutic intervention through transport of pharmacological agents to the involved tissue. It is commonly believed that a large number of systematically administered neurotherapeutics are not able to cross the BBB. This inefficient penetration of drugs into desired areas of the brain is recognized as a significant obstacle for the successful development of brain-targeted pharmaceutical compounds.

## Phage Display and Construction of Nanomaterial Libraries

Phage nanobiotechnology mainly relies on the versatile and revolutionary technology of phage display in which genetic engineering of the phage serves as a basis for introducing exogenous peptides and proteins onto the outer surface of the virion. Phage display is particularly characterized by physical linkage between the phenotype (the surface expressed peptide) and genotype (the DNA sequence that encodes that peptide) within the same viral particle. The surface functionalization of phage nanoparticles provides the possibility to construct phage display libraries. These nanomaterial libraries can be used for identifying and selecting pharmaceutical ligands with favorable characteristics against various biological targets. In this context, phage display represents potential to be used in the formulation of drug delivery systems. The construction of a library is achieved through making use of randomly generated DNA sequences and molecular biology methods. For this purpose, randomized oligonucleotides are spliced into the appropriate site of a gene that encodes one of the phage surface proteins. This leads to the display of a diversified library of peptides on the surface of phage nanoparticles. While the entire library contains an innumerable number of phage nanostructures, each phage displays multiple copies of a unique peptide sequence on its surface (Bakhshinejad et al., 2014). In principle, phage nanomaterial libraries harbor a wide collection of nanotubes decorated with diverse peptides whose composition will be the critical determinant of physicochemical hallmarks of the whole nanoparticle.

The affinity-based screening of phage display libraries can be performed straightforwardly against different targets in order to isolate peptides with tight affinity from a population – millions or even billions – of candidate ligands. The process of affinity-based screening of phage peptide libraries for identifying those with particular binding specificity to a given target is called panning. In panning, phage library is incubated with the desired target for a specified period of time. Among phage population of the library, some phages whose surface displayed peptides are able to interact with the target with greater affinity will adhere. The unbound phages are washed away and the remaining tightly bound phages are recovered (through alteration of pH or salt concentration) from the target. The eluted fraction is used to infect host bacterial cells for amplifying the target-bound phages. On the whole, this procedure is repeated multiple times (generally 3 to 5 rounds) in order to effectively enrich the highest affinity binders. Finally, target-specific binding peptides are identified by DNA sequencing of the bound phages (Bakhshinejad et al., 2014). The target that is exposed to the library through panning can have a biological or non-biological identity. For example, cells in culture can be exploited as the bait for capturing cell-specific peptides. Even with the heterogeneous characteristics of the cell surface, cell biopanning has exhibited dramatic potential in the selection of cell binding ligands for numerous cell types. Furthermore, a negative selection can be used to strip library of nonspecific peptides. These nonspecific peptide ligands might bind to surface receptors that are common among different cell types leading to spurious final results.

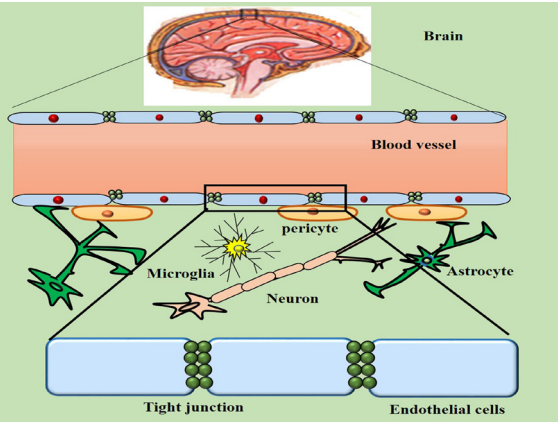


**Figure 1 The major anatomical and functional components of the blood brain barrier.**
Adjacent endothelial cells in the wall of blood vessels surrounding the brain have tight junctions that form a hardly passable and continuous barrier between the blood vessel and the brain. BBB also harbors astrocytes, pericytes, and microglia.

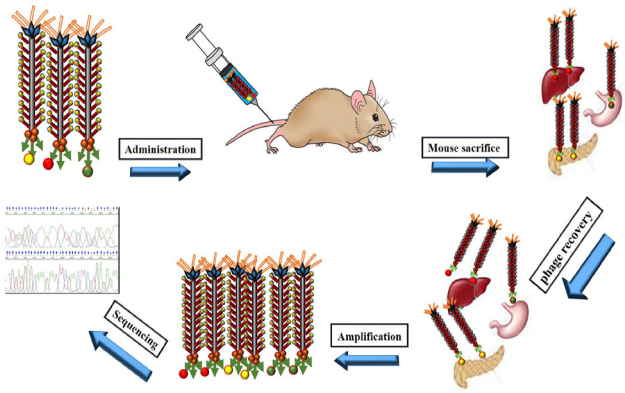


**Figure 2 *In vivo* panning of phage peptide libraries.**
The screening of a phage peptide library can be performed in a living organism. *In vivo* panning involves the following steps: systemic administration of the phage library into the mouse, animal sacrifice and extraction of the desired tissues/organs, recovery of the tissue/organ-bound phages, amplification of the recovered phages and phage genome sequencing.

Filamentous phages of the Ff class in particular M13 are considered as the main phage display platforms. As phage nanobiotechnology is strictly tied to phage display technology, therefore filamentous phages have become major viral tools in nanobiotechnology. Alternative phages including lambda, T4, T7 and P4 have served as display systems as well as for constructing peptide libraries. The assembly process of these phages and subsequent folding of displayed peptides occur thoroughly in the cytoplasm (Castagnoli et al., 2001). For this reason, these systems are appropriate for display of large, globular and hydrophobic or poorly soluble fusions. Although having a two-decade long history, libraries constructed based on these alternative phage display systems have not achieved commercial status and played a minor role in phage display research.

Filamentous phages boast some dramatic advantages over other phage types for use in phage display and accordingly in nanobiotechnology. Filamentous phages are flexible nanofibers. While the phage diameter is unvarying, the virion length may change in accordance with the length of the packaged DNA. This is achieved through increase or decrease of pVIII subunits. Therefore, the genome of filamentous phages can accommodate larger sequences. Long, rod-like and highly anisotropic shape leads filamentous phages to exhibit liquid-crystalline properties. The capacity to create one- and two-dimensional crystalline arrays in suspensions enables them to form ornately ordered self-assembled films and fibers (Yang et al., 2013). Also, rod-like nanoparticles generally possess a higher aspect ratio. Increased length-to-diameter ratio of filamentous phages results in more efficient cellular attachment and ensuing membrane penetration (Kolhar et al., 2013). This better cellular internalization may be due to the involvement of a higher number of ligand-receptor interactions that lead to a more considerable contact of elongated phages with the cell membrane.

While spherical nanoparticles have a tendency to remain in the center of blood vessels, rod-shaped nanoparticles show preference to margination and drifting toward the vessel wall (Toy et al., 2014). In this manner, rod-like phages may become able to more efficiently bind to receptors on the walls of blood vessels and subsequently extravasate, thereby bypassing the BBB. In line with this, the exploitation of synthetic microvascular networks that mimic natural vasculature indicated that rod-shaped polystyrene nanoparticles have higher avidity and selectivity than spherical ones to endothelial cells. Also, *in vivo* studies confirmed the increased specificity and vascular targeting of elongated nanoparticles to the brain endothelium (Kolhar et al., 2013). Although this study was performed on polystyrene nanoparticles, the rod shape-induced enhancement of brain endothelium targeting may unveil brain targeting potential of other rod-like nanoparticles such as filamentous phages.

## Phage Library Screening: Isolation of Brain-Homing Peptides

The screening of phage display libraries can be used as an efficient tool for obtaining peptides that home to the brain cells and tissues. This is achieved through both *in vitro* and *in vivo*.

*In vitro* panning has been demonstrated to be capable of identifying peptides that specifically bind to human brain tumor cells. Malignant glioma is known as one of the most fatal brain tumors. Despite advancements in the establishment of therapeutic approaches for glioma tumors, the presence of BBB and infiltrative properties of tumor cells pose challenges to the prognosis and treatment of glioma patients. These hurdles highlight the need to outline strategies for targeted delivery of therapeutic cargoes to glioma tumors. In line with this scenario, the screening of phage library led to the isolation of GL1 peptide that specifically interacts with human glioma cell lines as well as primary glioma cells obtained from human biopsy specimens. Also, when injected into the mouse through systemic route, GL1-bearing phages homed exclusively to human glioma xenograft in the right hemisphere of the mouse brain (Ho et al., 2010). This peptide represents potential to be used for selective targeting of imaging labels or anticancer therapeutics to glioma cells. Another study applied *in vitro* phage display to identify peptides that specifically bind to glioma stem cells (GSCs) (Beck et al., 2011). GSCs as a subpopulation of glioma cells are thought to be the major drivers of brain tumor initiation, progression, and recurrence. Recurrence occurs due to tumor resistance against different conventional therapies including irradiation and chemotherapy. Therefore, preferential targeting of GSCs is of particular relevance to the treatment of glioblastoma multiforme patients. This GSC-targeting peptide indicated selective binding affinity to many undifferentiated GSCs, but not to their differentiated counterpart cells. Targeting is achieved through binding of the peptide to the Nestin protein. The administration of GSC-homing peptide into the tumor-bearing mice resulted in effective penetration into the animal brain, and specific accumulation in gliomas arising from subcutaneous and orthotopic (intracranially injected) implantation. The identification of GSC-selective peptides that target GSCs may provide a promising opportunity to establish novel diagnostic and therapeutic modalities for eradicating GSCs present in many types of human brain malignancies.

The panning of phage peptide libraries can also be carried out in a living organism (**Figure 2**). *In vivo* panning is a means to identify peptide ligands capable of homing to specific tissues or organs. During *in vivo* panning, phage peptide library is intravenously administered (primarily through the tail vein) into a mouse and allowed to circulate. After a defined incubation time, the mouse is sacrificed and the desired target tissues or organs are extracted and homogenized in saline. The tissue/organ-associated phages are recovered and amplified through infecting bacterial host. The retrieved phages are plated to isolate plaques for DNA sequencing. This process is repeated with higher stringency by injecting the suspension of recovered phages into another mouse for an additional round of affinity selection (Babickova et al., 2013). Successive rounds of *in vivo* selection ultimately lead to the enrichment of a few number of peptide motifs with specific binding to a given tissue/organ. While non-specific phages turn to be distributed through the whole body of the animal, *in vivo* selection causes the clustering of highly selective peptide ligands in particular tissues. This *in vivo* approach has been reported to provide phage-displayed peptides that are able to traverse the BBB from systemic circulation. The *in vivo* screening of a cyclic 7-mer phage library was shown to yield a peptide specific to brain vascular receptors. This peptide, called PepC7, showed high efficiency in translocation into the mouse brain. *In vivo* optical imaging analysis revealed the brain-targeting capacity of this peptide (Li et al., 2012). Also, the peptide motif termed TGN was obtained from multiple rounds of *in vivo* screening of a 12-mer phage library. Compared with native phage, phage nanoparticles displaying the TGN peptide exhibited significantly higher efficiency in brain transport. Fluorescence microscopy revealed that TGN-bearing phages extensively accumulate in the third ventricle, lateral ventricle, periventricular region of the third ventricle, and cortex of the mouse brain (Li et al., 2011). Brain-targeting peptide motifs such as PepC7 and TGN – that have been identified *in vivo* – represent huge potential to develop efficient targeted platforms for the delivery of drugs as well as imaging agents to the brain.

These peptides can also be used for the discovery of new receptors or markers with potential application in brain targeting.

There is a considerable body of evidence in the literature – some mentioned here - on the use of filamentous phage-based libraries in order to identify brain-targeting peptides *in vivo*. These findings highlight the feasibility of using filamentous phages to cross the BBB. Nevertheless, there is a paucity of such type of information practically supporting the capacity of phages of other morphologies in navigating through the BBB.

## Phage Peptide Libraries: Development of Brain-targeted Drug delivery Nanocarriers

Phage display with its huge capacity in ligand identification can be used to obtain ideal moieties for surface functionalization of nanocarriers. Peptides derived from phage display libraries create new opportunities for the development of BBB-crossing brain-targeted drug delivery platforms. The previously mentioned TGN peptide – isolated from phage display peptide library- was conjugated to the surface of PEG-PLGA nanoparticles, while NAP (a peptide drug able to inhibit $A\beta_{1-40}$ fibril formation) was entrapped in the TGN-coupled PEG-PLGA nanocarriers. The neuroprotective effect of TGN-bearing NAP-loaded nanocarrier was demonstrated in an *in vivo* mouse model of Alzheimer's disease by using Morris water maze experiment, tissue histology and biochemical tests. Furthermore, morphological damage and Aβ plaques were not detected in the hippocampus and cortex treated with the TGN-targeted nanocarrier. The results revealed that TGN modification can protect drugs such as NAP from enzymatic degradation *in vivo* and efficiently mediate drug transport into the mouse brain after intravenous administration (Li et al., 2013). Also, the attachment of peptide ligands selected through combined *in vitro* /*ex vivo* phage display screenings to the surface of doxorubicin-loaded liposome nanocarriers led to a significantly reduced tumor volume and enhanced survival in the preclinical murine models of human neuroblastoma (Loi et al., 2013). The capability of this peptide-targeted drug delivery nanocarrier in counteracting NB progression in animal models demonstrates the efficacy of this system in crossing the BBB, thereby paving the way for the development of brain tumor-targeted drug delivery systems.

## Conclusion and Future Perspective

The exploitation of nanoscale platforms for bypassing the BBB has been introduced as an emerging concept in the territory of brain drug delivery. These nanostructured systems can be selectively targeted toward target sites in the body (for example the brain tissue). To minimize nonselective drug transport to non-target tissues and organs as well as improve drug delivery across the BBB, the surface chemistry of nanocarriers can be modified with cell- and tissue-specific peptides. Phage display libraries are known to be a rich source for the discovery of these homing peptides. The enormous capacity of phage display library as a powerful technology has bridged disciplinary boundaries and guided this screening strategy into diverse specialties ranging from materials to medical sciences. The presence of viral particles with nanosclae dimensions and properties in phage libraries has introduced them into the emerging field of nanobiotechnology. These unique properties of rod-shaped bacterial viruses have enabled phage researchers to develop commercial peptide libraries based on filamentous phages in particular M13 with extensive applications for panning studies. The commercial success of filamentous phage peptide libraries within the last several years has broadened the potential scope of phage display in nanomedicine. Furthermore, some methodological innovations such as development of landscape libraries have provided new perspectives to the use of phage-based combinatorial peptide libraries for selecting tumor-avid ligands.

Within the framework of nanobiotechnology, phage nanomaterial libraries can be adapted for the development of phage-derived nanometric-sized materials. When surface functionalized with selective peptide ligands, nanoparticulate carriers become capable of preferential binding to their well-matched receptors on the luminal side of endothelial cells of BBB. This approach, also called molecular Trojan horse strategy, lays a foundation to harness the power of receptor-mediated endocytosis for brain targeting of therapeutics. Recent findings highlight the fact that phage display is a powerful strategy for developing highly efficient targeted drug delivery platforms. The huge potential of this technology paves the way for the development of sophisticated and efficient brain-homing drug delivery systems in the future.

**Acknowledgments:** *We acknowledge the research deputy of the University of Tabriz due to kind supports.*

## References

Babickova J, Tothova L, Boor P, Celec P (2013) In vivo phage display--a discovery tool in molecular biomedicine. Biotechnol Adv 31:1247-1259.

Bakhshinejad B, Karimi M, Sadeghizadeh M (2014) Bacteriophages and medical oncology: targeted gene therapy of cancer. Med Oncol 31:110.

Beck S, Jin X, Yin J, Kim SH, Lee NK, Oh SY, Kim MK, Kim EB, Son JS, Kim SC, Nam DH, Kang SK, Kim H, Choi YJ (2011) Identification of a peptide that interacts with Nestin protein expressed in brain cancer stem cells. Biomaterials 32:8518-8528.

Bicker J, Alves G, Fortuna A, Falcao A (2014) Blood-brain barrier models and their relevance for a successful development of CNS drug delivery systems: a review. Eur J Pharm Biopharm 87:409-432.

Bradbury M (1979) The concept of a blood-brain barrier. Hoboken, NJ: Wiley.

Castagnoli L, Zucconi A, Quondam M, Rossi M, Vaccaro P, Panni S, Paoluzi S, Santonico E, Dente L, Cesareni G (2001) Alternative bacteriophage display systems. Comb Chem High Throughput Screen 4:121-133.

Ho IA, Hui KM, Lam PY (2010) Isolation of peptide ligands that interact specifically with human glioma cells. Peptides 31:644-650.

Kolhar P, Anselmo AC, Gupta V, Pant K, Prabhakarpandian B, Ruoslahti E, Mitragotri S (2013) Using shape effects to target antibody-coated nanoparticles to lung and brain endothelium. Proc Natl Acad Sci U S A 110:10753-10758.

Li J, Feng L, Fan L, Zha Y, Guo L, Zhang Q, Chen J, Pang Z, Wang Y, Jiang X, Yang VC, Wen L (2011) Targeting the brain with PEG-PLGA nanoparticles modified with phage-displayed peptides. Biomaterials 32:4943-4950.

Li J, Zhang Q, Pang Z, Wang Y, Liu Q, Guo L, Jiang X (2012) Identification of peptide sequences that target to the brain using in vivo phage display. Amino Acids 42:2373-2381.

Li J, Zhang C, Fan L, Jiang X, Chen J, Pang Z, Zhang Q (2013) Brain delivery of NAP with PEG-PLGA nanoparticles modified with phage display peptides. Pharm Res 30:1813-1823.

Loi M, Di Paolo D, Soster M, Brignole C, Bartolini A, Emionite L, Sun J, Becherini P, Curnis F, Petretto A, Sani M, Gori A, Milanese M, Gambini C, Longhi R, Cilli M, Allen TM, Bussolino F, Arap W, Pasqualini R, et al. (2013) Novel phage display-derived neuroblastoma-targeting peptides potentiate the effect of drug nanocarriers in preclinical settings. J Control Release 170:233-241.

Redzic Z (2011) Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. Fluids Barriers CNS 8:3.

Toy R, Peiris PM, Ghaghada KB, Karathanasis E (2014) Shaping cancer nanomedicine: the effect of particle shape on the in vivo journey of nanoparticles. Nanomedicine (Lond) 9:121-134.

Yang SH, Chung WJ, McFarland S, Lee SW (2013) Assembly of bacteriophage into functional materials. Chem Rec 13:43-59.