Published in final edited form as:

Adv Drug Deliv Rev. 2018 May ; 130: 39–49. doi:10.1016/j.addr.2018.06.015.

Current state of *in vivo* panning technologies: designing specificity and affinity into the future of drug targeting

Heather H. Gustafson, Audrey Olshefsky, Drew L. Sellers, Meilyn Sylvestre, and Suzie H. Pun*

Department of Bioengineering and Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA 98195, United States

Abstract

Targeting ligands are used in drug delivery to improve drug distribution to desired cells or tissues and to facilitate cellular entry. *In vivo* biopanning, whereby billions of potential ligand sequences are screened in biologically-relevant and complex conditions, is a powerful method for identification of novel target ligands. This tool has impacted drug delivery technologies and expanded our arsenal of therapeutics and diagnostics. Within this review we will discuss current *in vivo* panning technologies and ways that these technologies can be improved to advance next-generation drug delivery strategies.

Graphical Abstract

Keywords

Biopanning; drug delivery; peptides; aptamers; viral capsids; *in vivo*

1. Introduction

1.1 Biopanning: A platform to identify novel target ligands

Since its inception in 1985, biopanning has proven to be a powerful method for enhancing the rapid discovery of novel biological ligands for a variety of applications including, but not limited to, drug discovery, delivery and molecular biology[1–5]. The initial biopanning

*Corresponding Author: spun@uw.edu, 3720 15th Ave NE, Foege N530P, Box 355061, Seattle, WA 98195.

principle harnessed recombinant DNA technology[6] and Sanger sequencing[7] to engineer and develop biological platforms to express and display recombinant peptide-libraries in biological systems, which allowed for high throughput screening of novel ligands to a specified target of interest[5, 8–10]. Since that time, techniques such as directed evolution and next generation sequencing have increased development of high-affinity ligands[5, 11–13]. Furthermore, recombinant libraries have expanded from peptides to include antibodies, RNA, DNA and viral capsids—all providing a fresh and comprehensive approach to identify novel ligands[4, 14–16].

1.2 Targeted Drug Delivery: A need for novel ligand development

Targeted delivery of drugs involves technologies that increase distribution of pharmacologic agents to desired locations in the body. Delivery vehicles can mitigate off-target drug actions that result in significant and dose-limited side effects[17–19]. There are two main strategies for targeted drug delivery: passive or active targeting[18, 20, 21]. Passive targeting strategies rely on the physicochemical properties (e.g. charge, size) of a carrier and the body's physiology to affect drug distribution[20, 21]. In contrast, active targeting employs specific molecular interactions between the drug carrier and target tissues or cells for delivery[18, 20]. Despite the theoretical advantage of targeting, few FDA approved drug-formulations utilize an active targeting approach (Table 1).

The most commercially successful class of targeted delivery methods are antibody-drug conjugates or peptide/protein-based systems, wherein cytotoxic drugs are conjugated to a targeting protein via cleavable linkers (Table 1)[19, 22, 23]. These therapeutics have had clinical success due to their ability to recognize target cells with high affinity, to limit off-target toxicity, and to circulate for long periods. However, many protein-based targeting platforms (e.g. antibodies) are limited by their difficulty transporting agents beyond the vasculature due to their size and high binding affinity[24–26]. Therefore, significant interest exists for the development of small protein/peptides and nucleic acid structures that have greater accessibility and tissue penetration properties. Moreover, these small peptide and nucleic acid agents can be designed for oral and transdermal delivery to increase disease state accessibility and improve delivery outcomes[27–30]. Thus, biopanning methods for identifying a variety of targeting ligands are vital for developing and designing effective active targeting-carriers for a broad range of disease applications and administration routes.

2. *In vivo* biopanning platforms for developing novel targeting ligands

The immune system has evolved a tremendously complex and efficient means to produce antibodies that recognize new and recurrent foreign antigens either by expanding clonal populations of B-cells or by processing and presenting new antigens for antibody production. Antibodies against the new infectious agent are then selected as part of the adaptive immune response and improved through affinity maturation[31]. Thus, the diversity of unique antibodies in humans is in the billions[31, 32]. Biopanning is a process that mimics antibody selection by screening large libraries for potential binding ligands and then engineering and refining the ligands to increase binding affinity and targeting[8, 33, 34]. Through biopanning, novel ligands can be identified for target receptors for use as molecular

targeting agents in drug delivery. In this review, we will discuss the current active clinically approved biopanned agents, the literature that outlines *in vivo* biopanning technologies, and strategies to design novel panning platforms to improve ligand discovery and validation.

2.1. In vivo biopanning as a tool to discover novel ligands

The success of any biopanning strategy relies heavily on the design and stringency of a selection scheme to maximize stringency and eliminate non-specific binders. All biopanning paradigms utilize a ligand library that contains an inherent coding method (e.g. affiliated nucleic acid or chromophores) for moiety identification and reproduction after recovery of potential binders. Libraries are panned against a series or combination of positive- or negative-selection “schemes” to enrich the capture of specific binders over weak non-specific binders[35, 36]. For example, bacteriophage libraries can be incubated initially with a non-specific cell-type to remove off-target phage-binding (i.e. negative selection strategies). After removal of non-specific binders, the recovered phage population is then incubated with the target cell-type and washed to ensure stringent selection of high-affinity binders (i.e. positive selection)[9, 37, 38]. Multiple cycles and combinations of positive- or negative-selection can be performed to enrich for binding-phage further. After recovery of the high-affinity binders, the bacteriophage DNA is sequenced to identify each binding-motif[37, 39]. Validation of specific binders from the recovered pool is generally the most challenging and time-consuming step in biopanning[11, 13, 40, 41]. In *in vivo* biopanning, negative selection occurs naturally and with significant stringency as off-target tissue and protein interactions eliminate non-specific ligands to enrich the recovery of target-specific ligands and cellular receptors[42–44] (Figure 1). In theory, *in vivo* panning harnesses the heterogeneity of an intact biological system and improves the specificity of the identified ligand[43, 44]. Over the last 20 years, several *in vivo* panning display technologies and strategies have been developed. This section summarizes and highlights lessons learned.

2.2. In vivo Phage Display

The success of *in vivo* biopanning display is dependent on the library diversity and the stability of the panning platform. Increases in diversity increases overall hit rates and enhances novel outputs. Enhanced stability increases hit recovery and allows diversification of biological applications. We will outline within this review the current panning platforms each with their own current diversification and stability issues (Table 2).

2.2.1. Bacteriophage library platforms—Bacteriophage, or phage, libraries are generated through integration of a variable peptide sequences or proteins/antibodies onto a coat protein of bacteriophages[4, 50]. The phage platform incorporates both the displayed motif and the DNA encoding information into a single compact construct, allowing for simultaneous panning and subsequent sequencing of massively diverse libraries[8, 9]. For peptide libraries, the most commonly used phage vector is the M13 phage; the foreign peptide sequence, generally 7–12 amino acids in length, can be fused to minor pIII (3–5 copies of the peptide sequence) or major pVIII (2700 copies of the peptide) coat proteins[51]. It was discovered early on in phage biopanning that fusion to the pVIII reduces specificity of the isolated peptide sequence due to the selection of low affinity peptide binders that benefit from the increased avidity that results from high copy display [51]. In

contrast, libraries displayed on pIII at 3–5 copies per phage generally yield peptide binders with higher affinity. Thus, commercial libraries are generally constructed with a large diversity ($\sim 10^9$) of peptide sequences fused to the pIII coat protein of a filamentous M13 phage. The pIII display technology has been used to fuse antibody and small protein fragments [3, 5, 9]. There are a few examples of *in vivo* selection phage displaying antibody or protein fragments[52–55]. Other phage platforms include T7, T4 and λ bacteriophage; however, M13 phage is most commonly used due to its single coat fusion site, and ease of purification[51]. However, it has been demonstrated that the complex purification systems with T7- and T4-lytic phage allow for increased library diversity[56]. Moreover, T7 and T4-phage are small (~ 60 nm diameter) compared to M13 (~ 900 nm, length) and have been shown to have greater tissue penetration *in vivo*, enhancing delivery to M13-inaccessible sites such as the liver and brain [47, 57]. Lytic phage is also thought to have increased retention and reduced degradation in lysosomes, which might increase sequence yields in *in vivo* panning[56]. Despite these advantages the majority of *in vivo* panning studies have utilized recombinant, commercially available M13 peptide phage libraries.

2.2.2. Applications of *in vivo* phage display in humans—Initial *in vivo* biopanning efforts utilized phage libraries administered intravascularly and resulted in identification of a wide array of peptide “vascular zip codes” that target vasculature-specific ligands[42, 58] (Table 3). Further, peptide zip codes were shown to target agents directly to an organ or disease-associated vasculature[27, 59–66]. Following the success of biopanning in rodents, a similar phage-display method was applied in human biopanning screens. The first *in vivo* human panning experiment to map the human vasculature was conducted on a patient declared dead via brain-based determinations and results published in 2002 [67]. Subsequently, similar studies were performed with cancer patients whereby phage was recovered from biopsy specimens after intravenous administration of libraries [68, 69]. These later human studies recovered peptides recognizing targets commonly associated with tumor-related vascular targets such as interleukins, annexin, cathepsins, and prostaglandins[67–69]. Considering the vast amount of data generated from these early human vascular screens, one could suggest that it may be worth revisiting the human *in vivo* biopanning strategies to take full advantage of next-generation sequencing technologies and bioinformatics tools, as well as other routes of administration.

2.2.3. Limitations to *in vivo* phage display: tissue distribution and circulation half-life—As discussed, intravenous administration of M13 phage libraries in animals and humans led to the discovery and characterization of peptide zip codes that target organ-specific vasculature and tumor-binding peptides for drug delivery applications[27, 42, 58, 67]. Bacteriophage are inherently stable in biological milieus, a useful trait for productive *in vivo* selection. Still, *in vivo* phage-display biopanning methods are limited in the reach of the application to target sites outside of the vasculature or vasculature-leaky disease sites due to limited extravasation and penetration of the M13 phage. While alternative display technologies with smaller size such as covalent display technologies such as polyosome, ribosome or RNA-peptide display, could in theory distribute more broadly into tissues, these agents have not been successfully designed to withstand the harsh conditions of an intact biological system[70, 71]. The smaller T7 phage systems might reach more potential targets

in vivo through increased accessibility, but trends in biopanning strategies (away from lytic phage) would suggest other process-related limitations complicate the use of T7- and T4-phage.

In addition to the size-based tissue exclusion, M13 biopanning is limited by a relatively short circulation half-life (4 hrs), which is reduced significantly for recombinant M13-based libraries (<20 min), likely due to a combination of reduced *in vivo* stability, increased biological interactions, and increased internalization rate to cells[45, 72]. Furthermore, after internalization, M13 phage are degraded within lysosomes in 30 mins[45, 72]. T7-phage have a faster clearance, with half-lives less than 20 min for both wildtype and peptide-modified phage [46, 73]. Indeed, all phage are recognized readily by the immune system and sequestered within immunological relevant organs[46]. It is worth noting that in animals with a compromised immune system, phage exhibit an increased circulation potential, like due to the reduced recognition of phage through B-cell interactions [46, 72]. Nonetheless, engineering phage or other peptide display technologies to improve the circulation half-life and to limit degradation by mutating immunogenic properties of the macromolecular structure would result in a more robust platform for *in vivo* selection.

2.2.4. In vivo phage panning from alternative administration routes—As intravenous administration of phage libraries resulted in rich identification of binding motifs within the vasculature, biopanning from alternative administration routes would be expected to provide access to alternative targets. Indeed, non-systemic administration of phage libraries have yielded targeting ligands for a wide variety of diseases[29, 30, 44, 74–77] (Table 4). Thus, the route of administration provides access to alternative targets that would normally be restricted by intravenous routes. For example, Wan et al., biopanned for phage that could transport to the brain after applying phage to the nasal epithelium of rats[74]. In a similar fashion, Sellers et al. administered phage by peripheral injection into the gastrocnemius, of mice, to identify ligands that targeted axonal import for delivery into the spinal cord 24 hours post-injection[44]. In these studies, peripheral administration with recovery at distal sites at time that eclipse several circulation half-lives (i.e. 1 hour), provides selection pressure for recombinant phage-sequences that demonstrate strong targeting potential.

2.3. In vivo viral capsid panning

2.3.1. Adeno-associated virus (AAV) panning—Viral capsids are intrinsically robust particles well-suited to *in vivo* library selection. Viral particles exhibit considerable stability *in vivo*, have diameters on the order of nanometers allowing for broad circulation, are easily cloned and produced, and have naturally evolved to encapsulate genetic information to allow for high-throughput viral clone identification[15, 78–80]. *In vivo* viral capsid panning has primarily been performed with adeno-associated viruses (AAVs), which are icosahedral, nonenveloped viruses belonging to the Parvoviridae family. AAVs package single-stranded DNA and are 20–25 nm in diameter allowing for effective transport and delivery. Recombinant AAVs (rAAVs) are of particular interest as vehicles for gene therapy due to their favorable safety profiles[81]. Moreover, rAAVs can be engineered to eliminate most viral DNA, as well as to target certain tissues and cell types[82]. While much of

biopanning has focused on AAVs, it is worth noting that CCMV, CPMV, and TMV are attractive viral particles because they do not naturally infect humans, their surfaces may be functionalized with peptides or small molecules, and their cargo may include imaging contrast agents or small molecule drugs[79, 83, 84]. Thus, future design of panning strategies might benefit from re-engineering these vector platforms.

Recombinant AAV particles with increased tissue tropisms can be engineered by generating highly diverse capsid libraries ($>10^6 - 10^8$ surface protein variants) and performing directed evolution *in vivo* to select for variants with desired biodistribution properties. There are several different approaches to generating viral capsid libraries as discussed below. As with phage display, *in vivo* AAV panning involves injecting the capsid library into laboratory animals and recovering the viral DNA from harvested tissues. Library variants that are recovered at a high frequency in tissues of interest compared to off-target tissues are enriched and re-injected for another round of *in vivo* panning. In addition to selecting for tissue tropism, rAAV biopanning has been used to discover clones that exhibit tissue-specific transduction by utilizing a Cre-based reporter to reveal tissue-specific increases in rAAV reporter protein expression[85–87]. In this strategy, rAAV particles carrying Cre-sensitive fluorescent reporter genes are subjected to *in vivo* selection in transgenic mice that express Cre recombinase in specific cell-types. A higher fluorescent signal correlates to increased transduction efficiency of a particular rAAV clone in a particular cell type. After multiple rounds of *in vivo* selection, rAAV variants with optimized characteristics including tissue tropism and transduction efficiency may be identified and further analyzed.

2.3.2. Directed evolution of randomly, semi-randomly, and rationally designed AAV libraries—Directed evolution of viral capsids for tissue targeting and transduction potency involves imposing selective pressures on large, random or semi-random capsid libraries[88]. This tool allows for rapid affinity and selectivity improvements, enhancing AAV-mediated delivery outcomes. One successful library approach utilizing this technique, has been to randomly shuffle *Cap* (capsid) genes from different AAV serotypes[89]. DNA shuffling has yielded rAAV particles with up to 13-fold increased gene transfer in heart muscle cells compared to preexisting AAVs, and particles with over 400-fold increased resistance to neutralizing antibodies[89, 90]. Libraries may also be generated in a semi-random fashion by targeted mutagenesis of *Cap* genes. For example, a rAAV with up to 15-fold increased transduction of astrocytes was developed by mutagenizing the capsid at loop regions performing *in vivo* library selection[91]. Peptide insertion, whereby peptides of random lengths and sequences are incorporated into the viral capsid surface, has also been used to engineer improved AAV tropism[16, 92]. Korbelen and colleagues recently used this library approach to engineer pulmonary tissue-specific rAAVs with high gene delivery efficiency[93]. Library diversity is a critical feature in increasing likelihood of successful selection. Thus, another approach to viral capsid panning is to utilize several different types of libraries in the same experiment. By combining four different libraries, a rAAV with 100-fold increased access to projection neurons compared to preexisting AAVs was engineered[85]. Future libraries may combine libraries multiplicatively rather than additively, allowing for even higher theoretical complexity. For example, after randomly shuffling *Cap* genes, random peptide insertions may be introduced into the same library.

Random library generation approaches yield many rAAV particles with reduced transduction efficiency or non-functional particles, reducing the effective library diversity. To address this limitation, rational design of viral capsid libraries can be performed utilizing known viral capsid structures and functional regions. The Schaffer group recently developed the SCHEMA library approach that was successfully used for *in vivo* panning[87]. In this approach, the SCHEMA algorithm[94] and recombination as a shortest path (RASPP) method[95] were applied to maximize both library diversity and structural conservation. After generating and screening a library of over 1.6 million rAAV variants, a novel variant was isolated that exhibited 60% transduction efficiency in adult neural stem cells, 24-fold higher GFP expression than AAV9, and an increased ability to evade neutralizing antibodies. This example highlights how rational modifications to libraries to selectively modify diversity can improve delivery and therapeutic outcomes and should be considered in *in vivo* panning design.

2.4. In vivo Aptamer Panning

2.4.1. Aptamers and aptamer selection—Nucleic acid aptamers are single-stranded oligonucleotides, typically 15–100 base pairs in length, that form three-dimensional structures capable of binding to targets with high specificity and affinity. Aptamer libraries used in biopanning generally contain 10^{13} – 10^{15} unique sequences, consisting of a 20–60 base pair random region flanked by fixed primer regions on the 5' and 3' ends. From this starting pool, DNA or RNA aptamers are selected through the iterative process of Systematic Evolution of Ligands by Exponential Enrichment (SELEX), involving cycles of incubation with the target and subsequent amplification and recovery of binding sequences[2, 96]. With successive cycles, high affinity aptamers are enriched until they dominate the library population[14, 36, 97]. The population of aptamers present after selection can be characterized by next generation sequencing (NGS), and individual aptamers can then be synthesized and investigated for binding behavior.

Aptamer ligands offer several advantages over conventional targeting peptides or antibodies. Aptamers can form a variety of complex secondary structures with comparable or superior binding affinity of antibodies (micromolar to picomolar), and they can bind to a wide breadth of targets, such as ions, small molecules, and proteins. Since aptamers are nucleic-based, they exhibit reduced immunogenicity, increased tissue penetration, facile manufacturing, and high shelf-life stability compared to peptides and proteins. Thus, aptamers are an attractive class of targeting agents for drug delivery. However, aptamers are highly susceptible to nuclease degradation and kidney filtration, resulting in limited circulation time and half-life *in vivo*. Multiple studies have demonstrate that these challenges can be mitigated by engineering chemical modification into the aptamer backbone or side groups, non-natural nucleotide substitutions, or end capping, and reducing susceptibility to endo- and exo-nucleases[98, 99], which have aided the development of *in vivo* aptamer panning strategies[36].

2.4.2. Library design for *in vivo* SELEX—There are several considerations involved in the design aptamer libraries for *in vivo* SELEX, including nucleotide type, library length, chemical modification, and length. Aptamer library lengths are typically between 20–80

base pair, while aptamers short or long random regions can successfully bind, those with longer random regions exhibit greater structure complexity[100] and increased library diversity at the expense of cost for production. Both RNA and DNA aptamer libraries have yielded binding sequences from *in vivo* panning. RNA libraries tend to increase library diversity and improve panning outcomes but usually at the expense of stability. Aptamers based on RNA, which is naturally single-stranded have a greater range of three dimensional structures compared with DNA, which can result in a higher binding-affinity [101]. However, RNA aptamers are also more expensive and require an extra step of reverse transcription prior to amplification.

2.4.3. Recent progress in *in vivo* SELEX—For drug delivery applications, *in vivo* biopanning has an inherent advantage over *in vitro* panning by providing the stringent degradation, transport, and complex competitive binding pressures present *in vivo* during the selection process. *In vitro* SELEX has been performed successfully against purified target proteins or even whole cells; however, these *in vitro* selected aptamers may fail to bind to the same target *in vivo*. This may be attributed to differences in selection conditions, as aptamer conformation and binding has been demonstrated to depend on pH, temperature and ionic conditions[102–105]. To address this shortcoming, *in vivo* SELEX can be performed in living animal models to identify binding constructs in the context of a representative disease environment.

Mi et al. published the first RNA aptamer identified via *in vivo* SELEX in 2010[106]. In this work, a random library of 2'-fluoropyrimidine modified RNA sequences was injected intravenously into hepatic colorectal cancer-bearing mice. This chemical modification increases aptamer stability through increased nuclease resistance, thus increasing potential target binding by enhancing aptamer circulation time. Fourteen cycles of SELEX were performed and an aptamer that bound to p68, an RNA helicase, with nanomolar affinity was identified[14, 106]. Since this original publication, this process has been adapted for use with human intrahepatic xenografts to identify RNA aptamers against human helicase DHX9[107].

In vivo SELEX has also been used to evolve aptamers capable of penetrating the blood brain barrier (BBB). Penetration of therapies across the BBB remains a challenge due to tight cell junctions and highly selective transport of molecules. Cheng et al. injected intravenously a 2'-fluoropyrimidine modified RNA library to identify an aptamer that could penetrate the BBB after being, one of which represented 50% of all sequences. Further characterization of the most highly enriched aptamer from round 22 revealed that it internalized by brain-vascular endothelial cells. The authors did note high levels of RNA aptamer sequences in clearance tissues (liver and kidney) which was attributed to the reticuloendothelial system. Additionally, since the *in vivo* SELEX strategy required over 20 rounds of selection, the authors recommended a combinatorial *in vitro* and *in vivo* selection if screening for specific disease phenotypes[108].

Library stability and circulation time can drastically affect selection efficiency. *In vivo* SELEX was carried out successfully using a polyethylene glycol (PEG)-modified 2' fluoropyrimidine RNA library to identify an aptamer that binds to and inhibits

proliferation of non-small-cell lung cancer (NSCLC) cells[109]. Libraries were conjugated with PEG in order to increase aptamer circulation time and reduce renal clearance by increasing molecular weight. A binding aptamer was emerged at cycle 8 of selection and accounted for 90% of the enriched RNA by cycle 11, thus requiring much fewer rounds of selection compared with other *in vivo* SELEX reports[106, 108]. The authors attributed this early emergence to the addition of PEG within the RNA pool and the increased circulation time, which eliminated non-specific binding[109]. This aptamer was further used to facilitate delivery of a chemotherapeutic drug. In another recent example, Shen and coworkers used a DNA thiophosphate-backbone-substituted[110] library *in vivo* SELEX in a breast cancer bone metastasis model to identify a DNA aptamer that accumulates in the tumor microenvironment[111]. After 10 rounds of selection from tumor tissue, the top occurring aptamer was tested and shown to bind to cancer and myeloid-derived tumor suppressor cells. Furthermore, aptamer-conjugated liposomal doxorubicin formulations were effective in inhibiting tumor growth compared to non-targeted liposomes[36, 106].

For successful *in vivo* aptamer panning, it is critical that aptamer libraries are able to infiltrate the target area for selection, extraction, and amplification. However, unmodified aptamers are highly susceptible to nuclease degradation, resulting in a short half-life and circulation time *in vivo*. Additionally, their small size contributes to their rapid renal filtration. To prevent degradation, aptamer libraries have been modified chemically to replace the 2' position with a fluoro (F), amino (NH₂), or O-methyl (OCH₃) group, and by capping the 3' end with inverted thymidine[99, 112]. While chemical modifications can impart improved stability and circulation, chemical modification to the aptamer backbone or side-chains can affect aptamer folding and binding. Nonetheless, these changes can be utilized in-SELEX or post-SELEX biopanning strategies. For the in-SELEX biopanning, sequences with the chosen modifications are used in the starting DNA or RNA library and undergo subsequent rounds of selection. However, modifications are limited to 2'-aminopyrimidines, 2'-fluoropyrimidines, 2'-O-methyl nucleotides, and locked nucleic acids (LNA). Mi, Cheng, and Wang all utilized in-SELEX modifications for their *in vivo* panning strategies[107–109, 111]. Recently, SOMAmer (slow off-rate modified aptamer) reagents have emerged. They contain modified nucleotides that have conjugated functional moieties, creating stable structures with high nuclease resistance and binding affinities[113]. For the post-SELEX strategy, chosen modifications are implemented within pre-selected aptamers at specific locations during chemical synthesis. However, these modifications can affect aptamer structure and folding and thus impact its binding behavior.

Additionally, unmodified aptamers are within the size range for renal excretion and are thus rapidly excreted by the kidneys. To mitigate this, aptamers are often modified by the addition of a bulky side group, such as PEG or cholesterol. This strategy increased the plasma circulation half-life of Macugen, an aptamer used to treat macular degeneration, from 9 hours to 12 hours[99]. Wang utilized a combinatorial approach of 2'-fluoropyrimidine and PEGylation in order to increase *in vivo* stability[109]. Aptamers can also be multimerized to create multivalent constructs above the renal molecular weight threshold. Borbas et al. designed a tetrameric aptamer against the MUC1 tumor marker. This construct had improved stability, tumor-retention, and pharmacokinetic properties compared to the

monovalent construct[114]. As such, a library particle system displaying multiple copies of each aptamer could be another approach for *in vivo* aptamer panning.

3. Future of panning

In vivo panning for novel ligands has enormous potential for targeted drug delivery. The platform inherently allows for simultaneous selection of molecules that are able to withstand harsh biological environments, and home to targets in a complex and heterogeneous milieu. However, the design of successful *in vivo* biopanning requires consideration of multiple design principles. Success in panning is dependent on both the diversity and stability of the library. Here we will discuss ways to both enhance these parameters as well as manipulate panning procedures to artificially augment current panning systems.

3.1. Increasing *in vivo* library stability

Library stability is necessary to recover bound ligands. Maintaining the stability of a library in a harsh biological environment has challenges and *in vivo* panning requires library exposure to multiple biological milieu, potentially impacting the diversity of the final library at the target site. Novel targets might be lost as a result of degradation. Ultimately the perfect library display platform would be engineered to withstand harsh biological milieu. The conditions a display platform must withstand during biological transport include enzymatic degradation, harsh shear force, dramatic pH changes including acidic endosome/lysosomal conditions following platform internalization into cells, etc. Some groups have begun to address some these challenges in *in vitro* panning systems. Rangel et al. incorporated a cell penetrating peptide, penetratin, on the on the pVIII major coat protein of M13 phage, enhancing intracellular release for subcellular organelle ligand selection[39]. It's possible strategies like these could be applied *in vivo*, allowing the library platform to avoid lysosomal degradation and improve ligand recovery. However, modulation to increase lysosomal stability likely does not eliminate loss of phage simply due to inherent physical transport limitations. Overcoming transport challenges is likely overcome through panning design.

3.1.1 Design of clever panning strategies to improve library recovery.—In order to increase the likelihood of completing a successful *in vivo* biopanning, careful consideration of the inherent biology of the target and/or disease state must be given to each strategy. The most successful targeting ligands have been discovered by *in vivo* panning that carefully considered the inherent disease state and a specific route of administration that would allow for increased exposure to the target. Incorporation of the disease state into the panning strategy inherently increases affinity and specificity of a ligand, through the incorporation of heterogeneity of diseased and “normal” target sites. Thoughtful routes of administration to exploit routes of delivery to disease tissue or the intended target could improve ligand selection through enhanced ligand presentation, by decreasing library degradation during transport to the target site, or by improving negative selection by passage through competitive non-specific binding targets. Such examples include Sellers et al. recent neurological panning strategy where we were able to harness biological transport within the peripheral nervous system from muscles to the spinal cord[44]. Through intramuscular

injections we were able to isolate and validate a novel peptide that helps to engage this transport pathway (Figure 2). This study highlights how biological processes can be integrated into panning design strategies. While strategies like these allow for increased target exposure it does not enhance the diversity of the starting library nor does it improve ligand affinity.

3.2 Increased *in vivo* library diversity

In vivo panning success achieved when a ligand with high affinity and specificity to a target site is discovered. While panning strategies can be modified to improve outcomes, in general increasing the number of panned ligands will inherently increase the statistical probability of a hit. As shown in Table 2, current *in vivo* display platforms have significant differences in absolute diversity. Many researchers have probed these platforms *in vitro* to increase diversity through synthetic modification, such as artificial nucleic or amino acids, as well as, the introduction of chemical functionality. Such examples include SOMAmers, which add non-natural functional groups to aptamers to more closely mimic protein interactions, and one-bead-one-compound libraries for peptide selection which can include non-natural amino acids which can reverse chirality[113, 115]}. These platforms have yielded high affinity motifs not necessarily found in nature, and can be combined with natural ligand libraries to improve diversity. These diversification techniques have been widely applied to *in vitro* selection but have not been either optimized or applied for *in vivo* panning. Increasing the synthetic tool sets could dramatically enhance *in vivo* panning target identification and outcome. These days, panning products are often assessed through next generation sequencing methods where ligand hits from each panning round are screened for sequence similarities and alignment[11, 116, 117]. After even the first round of *in vivo* panning, clear trends generally emerge within this analysis. This pattern recognition can be reinvested into subsequent rounds of panning to enhance library affinity, specifying diversity, a technique generally termed directed evolution.

3.2.1 Directed evolution to drive improved library hits.—Directed evolution techniques have been employed in viral and phage display in an *in vitro* context[5, 87]. These platforms significantly enhance specificity and affinity of ligands identified. However the leap has not yet made a dramatic impact on *in vivo* panning[5, 38, 87, 116, 118]. In directed evolution, following rounds of selection, patterns emerge. These motifs can be incorporated into subsequent panning rounds and affinity can be honed utilizing a deconvolution approach. The libraries can be generated either through iterative single positional scanning and/or multi-site mutational sublibrary scanning[51]. In both strategies, motif modifications are generally accomplished through site-specific mutagenesis or through random generation whereby motifs are fragmented and reassembled[51]. When individuals are refining library design for subsequent panning rounds, it might also be useful to scan bioinformatic databases to see if the motif identified have sequence homology with known native ligand binders. This could be particularly useful in phage display technology as sequence specificity of clinically translated sequences have been shown through these techniques to hone to three amino acid positions [68, 119, 120]. The beauty of panning is that multiple mutations can be generated in several libraries and be explored in a parallel fashion such that non-specific binders are rapidly removed and affinity honed

simultaneously (Figure 3). Unlike *in vitro* selection, incorporating this technique into *in vivo* selection allows for refinements to be made within the context of the real world application reducing time later for application optimization.

3.3 Novel display platforms.

The incorporation of both diversity and stability into a single, easy to use platform would be beneficial. The reason the vast majority of *in vivo* panning literature has used the M13 platform is because of its experimental ease of use. New protein and viral based platform technologies are also arising on the *in vivo* panning horizon. Synthetic nucleocapsids that were recently developed by the Baker and King groups might also be applied to *in vivo* panning[121]. These synthetic protein nanocages encapsulate their own genomes and are of comparable size to AAVs, allowing high-throughput production, broad circulation *in vivo*, and nucleic acid sequence-based identification. Furthermore, directed evolution strategies could be easily applied to this technology. Current generation synthetic nucleocapsids possess circulation half-lives comparable to M13 bacteriophage and have been engineered to display peptides for tagging and purification. With diameters < 30 nm, synthetic nucleocapsids are therefore an attractive future platform for *in vivo* panning.

4. Conclusions

In vivo panning has been relatively unexplored relative to *in vitro* panning since its inception in 1996. However, sequences identified by *in vivo* panning have moved quickly toward clinical use. The most well-known recovered *in vivo* panned sequences RGD[27] and NGR[122] have multiple on-going clinical trials both alone and in combination with therapeutics/diagnostics (Table 1) [123–125]. Advancements in *in vivo* panning strategies, through clever selection design, increased *in vivo* stability, and innovative directed evolution strategies, will launch our discoveries and improve delivery into the next 20 years

5. Acknowledgements.

The work was supported by NIH 1R01CA177272, 1R21NS099654, 1R21NS086500 and 1R01HL139007. HHG was supported by the Cardiovascular Pathology Training Grant (5 T32 HL 007312-37).

Abbreviations

(AAVs)	Adeno-associated viruses
(RASPP)	Recombination as a shortest path
(SELEX)	Systematic Evolution of Ligands by Exponential Enrichment
(NGS)	Next generation sequencing
(BBB)	Blood brain barrier
(SOMAmer)	Slow off-rate modified aptamer

6. References.

- [1]. Smith GP, Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, *Science*, 228 (1985) 1315–1317. [PubMed: 4001944]
- [2]. Ellington AD, Szostak JW, In vitro selection of RNA molecules that bind specific ligands, *Nature*, 346 (1990) 818–822. [PubMed: 1697402]
- [3]. McCafferty J, Griffiths AD, Winter G, Chiswell DJ, Phage antibodies: filamentous phage displaying antibody variable domains, *Nature*, 348 (1990) 552–554. [PubMed: 2247164]
- [4]. Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR, Making antibodies by phage display technology, *Annual review of immunology*, 12 (1994) 433–455.
- [5]. Esvelt KM, Carlson JC, Liu DR, A system for the continuous directed evolution of biomolecules, *Nature*, 472 (2011) 499–503. [PubMed: 21478873]
- [6]. Jackson DA, Symons RH, Berg P, Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*, *Proceedings of the National Academy of Sciences of the United States of America*, 69 (1972) 2904–2909. [PubMed: 4342968]
- [7]. Sanger F, Air GM, Barrell BG, Brown NL, Coulson AR, Fiddes CA, Hutchison CA, Slocumbe PM, Smith M, Nucleotide sequence of bacteriophage phi X174 DNA, *Nature*, 265 (1977) 687–695. [PubMed: 870828]
- [8]. Scott JK, Smith GP, Searching for peptide ligands with an epitope library, *Science*, 249 (1990) 386–390. [PubMed: 1696028]
- [9]. Clackson T, Hoogenboom HR, Griffiths AD, Winter G, Making antibody fragments using phage display libraries, *Nature*, 352 (1991) 624–628. [PubMed: 1907718]
- [10]. Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G, Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains, *Nucleic acids research*, 19 (1991) 4133–4137. [PubMed: 1908075]
- [11]. Liu GW, Livesay BR, Kacherovsky NA, Cieslewicz M, Lutz E, Waalkes A, Jensen MC, Salipante SJ, Pun SH, Efficient Identification of Murine M2 Macrophage Peptide Targeting Ligands by Phage Display and Next-Generation Sequencing, *Bioconjugate chemistry*, 26 (2015) 1811–1817. [PubMed: 26161996]
- [12]. Rouet R, Jackson KJL, Langley DB, Christ D, Next-Generation Sequencing of Antibody Display Repertoires, *Frontiers in immunology*, 9 (2018) 118. [PubMed: 29472918]
- [13]. Yang W, Yoon A, Lee S, Kim S, Han J, Chung J, Next-generation sequencing enables the discovery of more diverse positive clones from a phage-displayed antibody library, *Experimental & molecular medicine*, 49 (2017) e308. [PubMed: 28336957]
- [14]. Zhou J, Rossi J, Aptamers as targeted therapeutics: current potential and challenges, *Nature reviews. Drug discovery*, 16 (2017) 181–202. [PubMed: 27807347]
- [15]. Grimm D, Buning H, Small But Increasingly Mighty: Latest Advances in AAV Vector Research, Design, and Evolution, *Human gene therapy*, 28 (2017) 1075–1086. [PubMed: 28835125]
- [16]. Korbelen J, Trepel M, How to Successfully Screen Random Adeno-Associated Virus Display Peptide Libraries In Vivo, *Human gene therapy methods*, 28 (2017) 109–123. [PubMed: 28193101]
- [17]. Yoo JW, Irvine DJ, Discher DE, Mitragotri S, Bio-inspired, bioengineered and biomimetic drug delivery carriers, *Nature reviews. Drug discovery*, 10 (2011) 521–535. [PubMed: 21720407]
- [18]. Byrne JD, Betancourt T, Brannon-Peppas L, Active targeting schemes for nanoparticle systems in cancer therapeutics, *Advanced drug delivery reviews*, 60 (2008) 1615–1626. [PubMed: 18840489]
- [19]. Lambert JM, Morris CQ, Antibody-Drug Conjugates (ADCs) for Personalized Treatment of Solid Tumors: A Review, *Advances in therapy*, 34 (2017) 1015–1035. [PubMed: 28361465]
- [20]. Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC, Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology, *Advanced drug delivery reviews*, 66 (2014) 2–25. [PubMed: 24270007]

- [21]. Rabanel JM, Aoun V, Elkin I, Mokhtar M, Hildgen P, Drug-loaded nanocarriers: passive targeting and crossing of biological barriers, *Current medicinal chemistry*, 19 (2012) 3070–3102. [PubMed: 22612696]
- [22]. Sievers EL, Senter PD, Antibody-drug conjugates in cancer therapy, *Annual review of medicine*, 64 (2013) 15–29.
- [23]. Ruoslahti E, Tumor penetrating peptides for improved drug delivery, *Advanced drug delivery reviews*, 110–111 (2017) 3–12.
- [24]. Choi J, Credit K, Henderson K, Deverkadra R, He Z, Wiig H, Vanpelt H, Flessner MF, Intraperitoneal immunotherapy for metastatic ovarian carcinoma: Resistance of intratumoral collagen to antibody penetration, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12 (2006) 1906–1912. [PubMed: 16551876]
- [25]. Jain RK, Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors, *Cancer research*, 50 (1990) 814s–819s. [PubMed: 2404582]
- [26]. Thurber GM, Schmidt MM, Wittrop KD, Antibody tumor penetration: transport opposed by systemic and antigen-mediated clearance, *Advanced drug delivery reviews*, 60 (2008) 1421–1434. [PubMed: 18541331]
- [27]. Arap W, Pasqualini R, Ruoslahti E, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, *Science*, 279 (1998) 377–380. [PubMed: 9430587]
- [28]. Hsu T, Mitragotri S, Delivery of siRNA and other macromolecules into skin and cells using a peptide enhancer, *Proceedings of the National Academy of Sciences of the United States of America*, 108 (2011) 15816–15821. [PubMed: 21903933]
- [29]. Duerr DM, White SJ, Schluesener HJ, Identification of peptide sequences that induce the transport of phage across the gastrointestinal mucosal barrier, *J Virol Methods*, 116 (2004) 177–180. [PubMed: 14738985]
- [30]. Lee NK, Kim HS, Kim KH, Kim EB, Cho CS, Kang SK, Choi YJ, Identification of a novel peptide ligand targeting visceral adipose tissue via transdermal route by in vivo phage display, *J Drug Target*, 19 (2011) 805–813. [PubMed: 21999821]
- [31]. CAPRA JD, Janeway CA, Travers P, Walport M, *Immunobiology: the immune system in health and disease*, Garland Publishing 1999.
- [32]. Dunn-Walters DK, Ademokun AA, B cell repertoire and ageing, *Current opinion in immunology*, 22 (2010) 514–520. [PubMed: 20537880]
- [33]. Yao VJ, D'Angelo S, Butler KS, Theron C, Smith TL, Marchio S, Gelovani JG, Sidman RL, Dobroff AS, Brinker CJ, Bradbury ARM, Arap W, Pasqualini R, Ligand-targeted theranostic nanomedicines against cancer, *Journal of controlled release : official journal of the Controlled Release Society*, 240 (2016) 267–286. [PubMed: 26772878]
- [34]. Martins IM, Reis RL, Azevedo HS, Phage Display Technology in Biomaterials Engineering: Progress and Opportunities for Applications in Regenerative Medicine, *ACS Chem Biol*, 11 (2016) 2962–2980. [PubMed: 27661443]
- [35]. Giordano RJ, Cardó-Vila M, Lahdenranta J, Pasqualini R, Arap W, Biopanning and rapid analysis of selective interactive ligands, *Nature medicine*, 7 (2001) 1249.
- [36]. Wu YX, Kwon YJ, Aptamers: The “evolution” of SELEX, *Methods*, 106 (2016) 21–28. [PubMed: 27109056]
- [37]. Cieslewicz M, Tang J, Yu JL, Cao H, Zavaljevski M, Motoyama K, Lieber A, Raines EW, Pun SH, Targeted delivery of proapoptotic peptides to tumor-associated macrophages improves survival, *Proceedings of the National Academy of Sciences of the United States of America*, 110 (2013) 15919–15924. [PubMed: 24046373]
- [38]. Hawkins RE, Russell SJ, Winter G, Selection of phage antibodies by binding affinity. Mimicking affinity maturation, *J Mol Biol*, 226 (1992) 889–896. [PubMed: 1507232]
- [39]. Rangel R, Dobroff AS, Guzman-Rojas L, Salmeron CC, Gelovani JG, Sidman RL, Pasqualini R, Arap W, Targeting mammalian organelles with internalizing phage (iPhage) libraries, *Nat Protoc*, 8 (2013) 1916–1939. [PubMed: 24030441]
- [40]. Sefah K, Shangguan D, Xiong X, O'Donoghue MB, Tan W, Development of DNA aptamers using Cell-SELEX, *Nat Protoc*, 5 (2010) 1169–1185. [PubMed: 20539292]

- [41]. Ibsen KN, Daugherty PS, Prediction of antibody structural epitopes via random peptide library screening and next generation sequencing, *Journal of immunological methods*, 451 (2017) 28–36. [PubMed: 28827189]
- [42]. Pasqualini R, Ruoslahti E, Organ targeting in vivo using phage display peptide libraries, *Nature*, 380 (1996) 364–366. [PubMed: 8598934]
- [43]. Pasqualini R, Koivunen E, Ruoslahti E, Alpha v integrins as receptors for tumor targeting by circulating ligands, *Nature biotechnology*, 15 (1997) 542–546.
- [44]. Sellers DL, Bergen JM, Johnson RN, Back H, Ravits JM, Horner PJ, Pun SH, Targeted axonal import (TAXI) peptide delivers functional proteins into spinal cord motor neurons after peripheral administration, *Proceedings of the National Academy of Sciences of the United States of America*, 113 (2016) 2514–2519. [PubMed: 26888285]
- [45]. Molenaar TJ, Michon I, de Haas SA, van Berkel TJ, Kuiper J, Biessen EA, Uptake and processing of modified bacteriophage M13 in mice: implications for phage display, *Virology*, 293 (2002) 182–191. [PubMed: 11853411]
- [46]. Srivastava AS, Kaido T, Carrier E, Immunological factors that affect the in vivo fate of T7 phage in the mouse, *J Virol Methods*, 115 (2004) 99–104. [PubMed: 14656466]
- [47]. Ludtke JJ, Solloff AV, Wong SC, Zhang G, Wolff JA, In vivo selection and validation of liver-specific ligands using a new T7 phage peptide display system, *Drug Deliv*, 14 (2007) 357–369. [PubMed: 17701525]
- [48]. Rohloff JC, Gelinas AD, Jarvis TC, Ochsner UA, Schneider DJ, Gold L, Janjic N, Nucleic Acid Ligands With Protein-like Side Chains: Modified Aptamers and Their Use as Diagnostic and Therapeutic Agents, *Molecular therapy. Nucleic acids*, 3 (2014) e201. [PubMed: 25291143]
- [49]. Ying Y, Muller OJ, Goehring C, Leuchs B, Trepel M, Katus HA, Kleinschmidt JA, Heart-targeted adeno-associated viral vectors selected by in vivo biopanning of a random viral display peptide library, *Gene therapy*, 17 (2010) 980–990. [PubMed: 20393510]
- [50]. Smith GP, Petrenko VA, Phage Display, *Chemical reviews*, 97 (1997) 391–410. [PubMed: 11848876]
- [51]. Falciani C, Lozzi L, Pini A, Bracci L, Bioactive peptides from libraries, *Chem Biol*, 12 (2005) 417–426. [PubMed: 15850978]
- [52]. Hoogenboom HR, Overview of antibody phage-display technology and its applications, *Methods in molecular biology*, 178 (2002) 1–37. [PubMed: 11968478]
- [53]. Johns M, George AJ, Ritter MA, In vivo selection of sFv from phage display libraries, *Journal of immunological methods*, 239 (2000) 137–151. [PubMed: 10821955]
- [54]. Deramchia K, Jacobin-Valat MJ, Vallet A, Bazin H, Santarelli X, Sanchez S, Dos Santos P, Franconi JM, Claverol S, Bonetto S, Clofent-Sanchez G, In vivo phage display to identify new human antibody fragments homing to atherosclerotic endothelial and subendothelial tissues [corrected], *The American journal of pathology*, 180 (2012) 2576–2589. [PubMed: 22521648]
- [55]. Valadon P, Garnett JD, Testa JE, Bauerle M, Oh P, Schnitzer JE, Screening phage display libraries for organ-specific vascular immunotargeting in vivo, *Proceedings of the National Academy of Sciences of the United States of America*, 103 (2006) 407–412. [PubMed: 16384919]
- [56]. Deng X, Wang L, You X, Dai P, Zeng Y, Advances in the T7 phage display system (Review), *Molecular medicine reports*, 17 (2018) 714–720. [PubMed: 29115536]
- [57]. Urich E, Schmucki R, Ruderisch N, Kitas E, Certa U, Jacobsen H, Schweitzer C, Bergadano A, Ebeling M, Loetscher H, Freskgard PO, Cargo Delivery into the Brain by in vivo identified Transport Peptides, *Scientific reports*, 5 (2015) 14104. [PubMed: 26411801]
- [58]. Pasqualini R, Ruoslahti E, Tissue targeting with phage peptide libraries, *Mol Psychiatry*, 1 (1996) 423. [PubMed: 9154238]
- [59]. Houston P, Goodman J, Lewis A, Campbell CJ, Braddock M, Homing markers for atherosclerosis: applications for drug delivery, gene delivery and vascular imaging, *FEBS letters*, 492 (2001) 73–77. [PubMed: 11248240]
- [60]. Michon IN, Hauer AD, von der Thusen JH, Molenaar TJ, van Berkel TJ, Biessen EA, Kuiper J, Targeting of peptides to restenotic vascular smooth muscle cells using phage display in vitro and in vivo, *Biochimica et biophysica acta*, 1591 (2002) 87–97. [PubMed: 12183059]

- [61]. Kolonin MG, Saha PK, Chan L, Pasqualini R, Arap W, Reversal of obesity by targeted ablation of adipose tissue, *Nature medicine*, 10 (2004) 625–632.
- [62]. Zhi M, Wu KC, Dong L, Hao ZM, Deng TZ, Hong L, Liang SH, Zhao PT, Qiao TD, Wang Y, Xu X, Fan DM, Characterization of a specific phage-displayed Peptide binding to vasculature of human gastric cancer, *Cancer biology & therapy*, 3 (2004) 1232–1235. [PubMed: 15492500]
- [63]. Lee TY, Lin CT, Kuo SY, Chang DK, Wu HC, Peptide-mediated targeting to tumor blood vessels of lung cancer for drug delivery, *Cancer research*, 67 (2007) 10958–10965. [PubMed: 18006841]
- [64]. Newton JR, Kelly KA, Mahmood U, Weissleder R, Deutscher SL, In vivo selection of phage for the optical imaging of PC-3 human prostate carcinoma in mice, *Neoplasia*, 8 (2006) 772–780. [PubMed: 16984734]
- [65]. Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, Hanahan D, Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis, *Cancer cell*, 4 (2003) 393–403. [PubMed: 14667506]
- [66]. Hoffman JA, Giraudo E, Singh M, Zhang L, Inoue M, Porkka K, Hanahan D, Ruoslahti E, Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma, *Cancer cell*, 4 (2003) 383–391. [PubMed: 14667505]
- [67]. Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ, Mintz PJ, Ardeli PU, Yao VJ, Vidal CI, Chen L, Flamm A, Valtanen H, Weavind LM, Hicks ME, Pollock RE, Botz GH, Bucana CD, Koivunen E, Cahill D, Troncso P, Baggerly KA, Pentz RD, Do KA, Logothetis CJ, Pasqualini R, Steps toward mapping the human vasculature by phage display, *Nature medicine*, 8 (2002) 121–127.
- [68]. Staquicini FI, Cardo-Vila M, Kolonin MG, Trepel M, Edwards JK, Nunes DN, Sergeeva A, Efsthathiou E, Sun J, Almeida NF, Tu SM, Botz GH, Wallace MJ, O'Connell DJ, Krajewski S, Gershenwald JE, Mollidrem JJ, Flamm AL, Koivunen E, Pentz RD, Dias-Neto E, Setubal JC, Cahill DJ, Troncso P, Do KA, Logothetis CJ, Sidman RL, Pasqualini R, Arap W, Vascular ligand-receptor mapping by direct combinatorial selection in cancer patients, *Proceedings of the National Academy of Sciences of the United States of America*, 108 (2011) 18637–18642. [PubMed: 22049339]
- [69]. Krag DN, Shukla GS, Shen GP, Pero S, Ashikaga T, Fuller S, Weaver DL, Burdette-Radoux S, Thomas C, Selection of tumor-binding ligands in cancer patients with phage display libraries, *Cancer research*, 66 (2006) 7724–7733. [PubMed: 16885375]
- [70]. FitzGerald K, In vitro display technologies - new tools for drug discovery, *Drug Discov Today*, 5 (2000) 253–258. [PubMed: 10825731]
- [71]. Zahnd C, Amstutz P, Pluckthun A, Ribosome display: selecting and evolving proteins in vitro that specifically bind to a target, *Nature methods*, 4 (2007) 269–279. [PubMed: 17327848]
- [72]. Zou J, Dickerson MT, Owen NK, Landon LA, Deutscher SL, Biodistribution of filamentous phage peptide libraries in mice, *Mol Biol Rep*, 31 (2004) 121–129. [PubMed: 15293788]
- [73]. Srivastava AS, Chauhan DP, Carrier E, In utero detection of T7 phage after systemic administration to pregnant mice, *Biotechniques*, 37 (2004) 81–83. [PubMed: 15283204]
- [74]. Wan XM, Chen YP, Xu WR, Yang WJ, Wen LP, Identification of nose-to-brain homing peptide through phage display, *Peptides*, 30 (2009) 343–350. [PubMed: 19007831]
- [75]. Chen Y, Shen Y, Guo X, Zhang C, Yang W, Ma M, Liu S, Zhang M, Wen LP, Transdermal protein delivery by a coadministered peptide identified via phage display, *Nature biotechnology*, 24 (2006) 455–460.
- [76]. Wu M, Pasula R, Smith PA, Martin WJ 2nd, Mapping alveolar binding sites in vivo using phage peptide libraries, *Gene therapy*, 10 (2003) 1429–1436. [PubMed: 12900757]
- [77]. Akita N, Maruta F, Seymour LW, Kerr DJ, Parker AL, Asai T, Oku N, Nakayama J, Miyagawa S, Identification of oligopeptides binding to peritoneal tumors of gastric cancer, *Cancer science*, 97 (2006) 1075–1081. [PubMed: 16984380]
- [78]. Singh P, Gonzalez MJ, Manchester M, Viruses and their uses in nanotechnology, *Drug development research*, 67 (2006) 23–41.
- [79]. Manchester M, Singh P, Virus-based nanoparticles (VNPs): platform technologies for diagnostic imaging, *Advanced drug delivery reviews*, 58 (2006) 1505–1522. [PubMed: 17118484]

- [80]. Paulk NK, Pekrun K, Zhu E, Nygaard S, Li B, Xu J, Chu K, Leborgne C, Dane AP, Haft A, Zhang Y, Zhang F, Morton C, Valentine MB, Davidoff AM, Nathwani AC, Mingozzi F, Grompe M, Alexander IE, Lisowski L, Kay MA, Bioengineered AAV Capsids with Combined High Human Liver Transduction In Vivo and Unique Humoral Seroreactivity, *Molecular therapy : the journal of the American Society of Gene Therapy*, 26 (2018) 289–303. [PubMed: 29055620]
- [81]. Keeler AM, ElMallah MK, Flotte TR, *Gene Therapy 2017: Progress and Future Directions, Clinical and translational science*, 10 (2017) 242–248. [PubMed: 28383804]
- [82]. Naso MF, Tomkowicz B, Perry WL 3rd, Strohl WR, Adeno-Associated Virus (AAV) as a Vector for Gene Therapy, *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy*, 31 (2017) 317–334.
- [83]. Bruckman MA, Randolph LN, VanMeter A, Hern S, Shoffstall AJ, Taurog RE, Steinmetz NF, Biodistribution, pharmacokinetics, and blood compatibility of native and PEGylated tobacco mosaic virus nano-rods and -spheres in mice, *Virology*, 449 (2014) 163–173. [PubMed: 24418549]
- [84]. Czapar AE, Zheng YR, Riddell IA, Shukla S, Awuah SG, Lippard SJ, Steinmetz NF, Tobacco Mosaic Virus Delivery of Phenanthriplatin for Cancer therapy, *ACS nano*, 10 (2016) 4119–4126. [PubMed: 26982250]
- [85]. Tervo DG, Hwang BY, Viswanathan S, Gaj T, Lavzin M, Ritola KD, Lindo S, Michael S, Kuleshova E, Ojala D, Huang CC, Gerfen CR, Schiller J, Dudman JT, Hantman AW, Looger LL, Schaffer DV, Karpova AY, A Designer AAV Variant Permits Efficient Retrograde Access to Projection Neurons, *Neuron*, 92 (2016) 372–382. [PubMed: 27720486]
- [86]. Deverman BE, Pravdo PL, Simpson BP, Kumar SR, Chan KY, Banerjee A, Wu WL, Yang B, Huber N, Pasca SP, Gradinaru V, Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain, *Nature biotechnology*, 34 (2016) 204–209.
- [87]. Ojala DS, Sun S, Santiago-Ortiz JL, Shapiro MG, Romero PA, Schaffer DV, In Vivo Selection of a Computationally Designed SCHEMA AAV Library Yields a Novel Variant for Infection of Adult Neural Stem Cells in the SVZ, *Molecular therapy : the journal of the American Society of Gene Therapy*, 26 (2018) 304–319. [PubMed: 28988711]
- [88]. Nance ME, Duan D, Perspective on Adeno-Associated Virus Capsid Modification for Duchenne Muscular Dystrophy Gene Therapy, *Human gene therapy*, 26 (2015) 786–800. [PubMed: 26414293]
- [89]. Yang L, Li J, Xiao X, Directed evolution of adeno-associated virus (AAV) as vector for muscle gene therapy, *Methods in molecular biology*, 709 (2011) 127–139. [PubMed: 21194025]
- [90]. Koerber JT, Jang JH, Schaffer DV, DNA shuffling of adeno-associated virus yields functionally diverse viral progeny, *Molecular therapy : the journal of the American Society of Gene Therapy*, 16 (2008) 1703–1709. [PubMed: 18728640]
- [91]. Koerber JT, Klimczak R, Jang JH, Dalkara D, Flannery JG, Schaffer DV, Molecular evolution of adeno-associated virus for enhanced glial gene delivery, *Molecular therapy : the journal of the American Society of Gene Therapy*, 17 (2009) 2088–2095. [PubMed: 19672246]
- [92]. Korbelen J, Hunger A, Alawi M, Sieber T, Binder M, Trepel M, Optimization of design and production strategies for novel adeno-associated viral display peptide libraries, *Gene therapy*, 24 (2017) 470–481. [PubMed: 28622288]
- [93]. Korbelen J, Sieber T, Michelfelder S, Lunding L, Spies E, Hunger A, Alawi M, Rapti K, Indenbirken D, Muller OJ, Pasqualini R, Arap W, Kleinschmidt JA, Trepel M, Pulmonary Targeting of Adeno-associated Viral Vectors by Next-generation Sequencing-guided Screening of Random Capsid Displayed Peptide Libraries, *Molecular therapy : the journal of the American Society of Gene Therapy*, 24 (2016) 1050–1061. [PubMed: 27018516]
- [94]. Voigt CA, Martinez C, Wang ZG, Mayo SL, Arnold FH, Protein building blocks preserved by recombination, *Nature structural biology*, 9 (2002) 553–558. [PubMed: 12042875]
- [95]. Endelman JB, Silberg JJ, Wang ZG, Arnold FH, Site-directed protein recombination as a shortest-path problem, *Protein engineering, design & selection : PEDS*, 17 (2004) 589–594.
- [96]. Tuerk C, Gold L, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, *Science*, 249 (1990) 505–510. [PubMed: 2200121]

- [97]. Wu Y, Sefah K, Liu H, Wang R, Tan W, DNA aptamer-micelle as an efficient detection/delivery vehicle toward cancer cells, *Proceedings of the National Academy of Sciences of the United States of America*, 107 (2010) 5–10. [PubMed: 20080797]
- [98]. Xiang D, Shigdar S, Qiao G, Wang T, Kouzani AZ, Zhou SF, Kong L, Li Y, Pu C, Duan W, Nucleic acid aptamer-guided cancer therapeutics and diagnostics: the next generation of cancer medicine, *Theranostics*, 5 (2015) 23–42. [PubMed: 25553096]
- [99]. Darmostuk M, Rimpelova S, Gbelcova H, Ruml T, Current approaches in SELEX: An update to aptamer selection technology, *Biotechnology advances*, 33 (2015) 1141–1161. [PubMed: 25708387]
- [100]. Stoltenburg R, Reinemann C, Strehlitz B, SELEX--a (r)evolutionary method to generate high-affinity nucleic acid ligands, *Biomolecular engineering*, 24 (2007) 381–403. [PubMed: 17627883]
- [101]. Germer K, Leonard M, Zhang X, RNA aptamers and their therapeutic and diagnostic applications, *International journal of biochemistry and molecular biology*, 4 (2013) 27–40. [PubMed: 23638319]
- [102]. Hianik T, Ostatna V, Sonlajtnerova M, Grman I, Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin, *Bioelectrochemistry*, 70 (2007) 127–133. [PubMed: 16725379]
- [103]. Chang AL, McKeague M, Smolke CD, Facile characterization of aptamer kinetic and equilibrium binding properties using surface plasmon resonance, *Methods in enzymology*, 549 (2014) 451–466. [PubMed: 25432760]
- [104]. Yakimovich OY, Alekseev YI, Maksimenko AV, Voronina OL, Lunin VG, Influence of DNA aptamer structure on the specificity of binding to Taq DNA polymerase, *Biochemistry. Biokhimiia*, 68 (2003) 228–235. [PubMed: 12693970]
- [105]. Smestad J, Maher LJ 3rd, Ion-dependent conformational switching by a DNA aptamer that induces remyelination in a mouse model of multiple sclerosis, *Nucleic acids research*, 41 (2013) 1329–1342. [PubMed: 23175609]
- [106]. Mi J, Liu Y, Rabbani ZN, Yang Z, Urban JH, Sullenger BA, Clary BM, In vivo selection of tumor-targeting RNA motifs, *Nature chemical biology*, 6 (2010) 22–24. [PubMed: 19946274]
- [107]. Mi J, Ray P, Liu J, Kuan CT, Xu J, Hsu D, Sullenger BA, White RR, Clary BM, In Vivo Selection Against Human Colorectal Cancer Xenografts Identifies an Aptamer That Targets RNA Helicase Protein DHX9, *Molecular therapy. Nucleic acids*, 5 (2016) e315. [PubMed: 27115840]
- [108]. Cheng C, Chen YH, Lennox KA, Behlke MA, Davidson BL, In vivo SELEX for Identification of Brain-penetrating Aptamers, *Molecular therapy. Nucleic acids*, 2 (2013) e67. [PubMed: 23299833]
- [109]. Wang H, Zhang Y, Yang H, Qin M, Ding X, Liu R, Jiang Y, In Vivo SELEX of an Inhibitory NSCLC-Specific RNA Aptamer from PEGylated RNA Library, *Molecular therapy. Nucleic acids*, 10 (2018) 187–198. [PubMed: 29499932]
- [110]. King DJ, Ventura DA, Brasier AR, Gorenstein DG, Novel combinatorial selection of phosphorothioate oligonucleotide aptamers, *Biochemistry*, 37 (1998) 16489–16493. [PubMed: 9843415]
- [111]. Liu H, Mai J, Shen J, Wolfram J, Li Z, Zhang G, Xu R, Li Y, Mu C, Zu Y, Li X, Lokesh GL, Thivyanathan V, Volk DE, Gorenstein DG, Ferrari M, Hu Z, Shen H, A Novel DNA Aptamer for Dual Targeting of Polymorphonuclear Myeloid-derived Suppressor Cells and Tumor Cells, *Theranostics*, 8 (2018) 31–44. [PubMed: 29290791]
- [112]. Zhou Y, Chi H, Wu Y, Marks RS, Steele TWJ, Organic additives stabilize RNA aptamer binding of malachite green, *Talanta*, 160 (2016) 172–182. [PubMed: 27591602]
- [113]. Wu D, Katilius E, Olivas E, Dumont Milutinovic M., Walt DR, Incorporation of Slow Off-Rate Modified Aptamers Reagents in Single Molecule Array Assays for Cytokine Detection with Ultrahigh Sensitivity, *Analytical chemistry*, 88 (2016) 8385–8389. [PubMed: 27529794]
- [114]. Borbas KE, Ferreira CS, Perkins A, Bruce JI, Missailidis S, Design and synthesis of mono- and multimeric targeted radiopharmaceuticals based on novel cyclen ligands coupled to anti-MUC1 aptamers for the diagnostic imaging and targeted radiotherapy of cancer, *Bioconjugate chemistry*, 18 (2007) 1205–1212. [PubMed: 17583928]

- [115]. Lam KS, Lebl M, Krchnak V, The “one-bead-one-compound” combinatorial library method, *Chemical reviews*, 97 (1997) 411–448. [PubMed: 11848877]
- [116]. Hu D, Hu S, Wan W, Xu M, Du R, Zhao W, Gao X, Liu J, Liu H, Hong J, Effective Optimization of Antibody Affinity by Phage Display Integrated with High-Throughput DNA Synthesis and Sequencing Technologies, *PloS one*, 10 (2015) e0129125. [PubMed: 26046845]
- [117]. Dias-Neto E, Nunes DN, Giordano RJ, Sun J, Botz GH, Yang K, Setubal JC, Pasqualini R, Arap W, Next-generation phage display: integrating and comparing available molecular tools to enable cost-effective high-throughput analysis, *PloS one*, 4 (2009) e8338. [PubMed: 20020040]
- [118]. Infante YC, Pupo A, Rojas G, A combinatorial mutagenesis approach for functional epitope mapping on phage-displayed target antigen: application to antibodies against epidermal growth factor, *MAbs*, 6 (2014) 637–648. [PubMed: 24589624]
- [119]. Pasqualini R, Vascular targeting with phage peptide libraries, *Q J Nucl Med*, 43 (1999) 159–162. [PubMed: 10429511]
- [120]. Rajotte D, Ruoslahti E, Membrane dipeptidase is the receptor for a lung-targeting peptide identified by in vivo phage display, *The Journal of biological chemistry*, 274 (1999) 11593–11598. [PubMed: 10206967]
- [121]. Butterfield GL, Lajoie MJ, Gustafson HH, Sellers DL, Nattermann U, Ellis D, Bale JB, Ke S, Lenz GH, Yehdego A, Ravichandran R, Pun SH, King NP, Baker D, Evolution of a designed protein assembly encapsulating its own RNA genome, *Nature*, 552 (2017) 415–420. [PubMed: 29236688]
- [122]. Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, *Cancer research*, 60 (2000) 722–727. [PubMed: 10676659]
- [123]. Joshi S, Chen L, Winter MB, Lin YL, Yang Y, Shapovalova M, Smith PM, Liu C, Li F, LeBeau AM, The Rational Design of Therapeutic Peptides for Aminopeptidase N using a Substrate-Based Approach, *Scientific reports*, 7 (2017) 1424. [PubMed: 28465619]
- [124]. Carter A, Integrins as target: first phase III trial launches, but questions remain, *Journal of the National Cancer Institute*, 102 (2010) 675–677. [PubMed: 20460633]
- [125]. Kapp TG, Rechenmacher F, Neubauer S, Maltsev OV, Cavalcanti-Adam EA, Zarka R, Reuning U, Notni J, Wester HJ, Mas-Moruno C, Spatz J, Geiger B, Kessler H, A Comprehensive Evaluation of the Activity and Selectivity Profile of Ligands for RGD-binding Integrins, *Scientific reports*, 7 (2017) 39805. [PubMed: 28074920]

Figure 1.
In vivo phage panning schematic

Figure 2. Improve panning strategies to take advantage of inherent biology. Here, Sellers et al. capitalized on the transport from muscles to the spinal cord to bypass the blood brain barrier [44]

Figure 3. Directed evolution to improve affinity and specificity. Here, high diversity libraries are injected *in vivo*, selected and amplified. Selected variants are used to develop re-diversified libraries via fragmentation, reassembly and mutation. These libraries are used to improve affinity in subsequent panning rounds.

Table 1:

Pharmaceutical products with active drug targeting that are either FDA approved or in late-stage clinical trials

Biopanning method	Native Physiological Function	Clinical Application(s)	Target(s)	Trade Names	Development
Phage Display (Antibody)	Immunoglobulin, pathogen neutralization	Cancer	CD33, CD30, HER2	Mylotarg (Gemtuzumab ozogamicin), Adcetris (Brentuximab vedotin), Kadcyla (Trastuzumab emtansine)	Antibody Conjugated to Chemotherapeutic Drug (Pfizer/ Wyeth, Seattle Genetics/ Millennium/ Takeda, Genentech and Roche)
Phage Display (Kunitz Domain: 90 AA modifications in-between beta sheets)	Protease inhibitor (e.g. APP, TFPI)	Hereditary Angioedema	Kallirkein	Kalbitor (Ecallantide)	Macromolecular active drug discovered through phage display. (Shire)
Phage Display (Monobody: 90 AA modifications in-between beta sheets)	Fibrinectin type III domain	Glioblastoma	VEGFR2	Angiocept (Pegdinetanib)	Macromolecular active drug discovered through phage display. (Bristol-Myers Squibb)
Phage Display (Darpins: 24 ankyrin repeats)	Ankyrin, a membrane protein that anchors proteins to the actin cytoskeleton	Macular degeneration	VEGF-A	Abicipar	Macromolecular active drug discovered through phage display. (Molecular Partners AG)
Phage Display (Peptides, 7-12 residues)	Biologically active small molecules	Cancer	SSTR2, α_v integrins, CD13	Lutathera (Lutetium-Lu77-Dotatate), Cilengitide (RGD alone)/ Fluciclatide (RGD in combination with F18), Zafiride (NGR-TNF α)	Peptide radioisotope conjugate, peptide alone/peptide radioisotope conjugate, peptide cytokine conjugate. (Advanced Accelerator Applications/ Novartis, Merck/GM Healthcare, Adis Insight)

Table 2:Current *in vivo* Panning Platforms

Panning platform	Library type	Diversity	Typical number of selection rounds	Size	<i>In vivo</i> stability
Bacteriophage	Peptide	~10e9 (M13) to ~10e11 (T7)	3–5 rounds	60 nm for T7, 880 nm x 9 nm for M13	~4 hrs for unmodified and ~20 mins for peptide displayed M13[45], ~10 mins T7[46], increases after modification to reduce immune recognition [47]
	Antibody	~10e8	2–4 rounds		
Aptamer libraries	RNA and DNA	10e10- to 10e60 (depends on length, 4^n)	> 15 rounds (without PEG modification)	~10 kDa [48]	Low without modification
	Somamer	N/A (added post-SELEX)	N/A (added post-SELEX)	Depends on modification. I.e. PEG --> 40 kDa, NFkB-aptamer ~110 kDa[48]	High, but depends on modification
Virus	Virus	10e6 – 10e8	2–5 rounds	25 nm diam.	High
	Displayed peptide	Theoretical 10e8	3–5 rounds	~28 nm diam.	High but slightly reduced compared to unmodified[49]

Table 3.

Vascular targeting ligand zip codes discovered by *in vivo* phage display.

Target	Disease Target	Sequence	Reference
Atherosclerosis	Vascular accessible atherosclerotic plaque	CLVEAYPGLSVRSC	Houston 2001
Cancer Vasculature	Lung Cancer vasculature	SVSVGMKPSRP	Lee 2007
Obesity	Adipose vascular	CKGGRAKDC	Kolonin 2004
Cancer Vasculature	Gastric Cancer Vasculature	CGNSNPKSC	Zhi 2004
Atherosclerosis	Vascular smooth muscle cells	CNIWGVVLSWIGVFPEC	Michon 2002
Cancer Vasculature	Prostate Cancer	IAGLATPGWSHWLAL	Newton 2006
Cancer Vasculature	Breast Cancer	CDCRGDCFC	Arup 1998
Cancer Vasculature	Pancreatic cancer	CRGRRST	Joyce 2003
Cancer Vasculature	Squamous cell carcinoma.	CSRPRRSEC	Hoffman 2003

Table 4.

Alternative paradigms for non-systemic biopanning strategies.

<i>In vivo</i> Phage Panning Delivery Mechanism	Disease Target	Citation
Intranasal	Neurological Disorders	Wan 2009
Intramuscular	Neurological Disorders	Sellers 2016
Transdermal delivery	Diabetes	Chen 2006
Transdermal delivery	Obesity	Lee 2011
Intratracheal	Respiratory Disorders	Wu 2003
Oral	Intestinal Disorders	Duerr 2004
Intraperitoneal	Gastric metastases	Akita 2006

Table 5:*In vivo* SELEX

Aptamer library	Disease Model	Target	Affinity	Library Modification	Random Region (bp)	Library Circulation Time
RNA (Mi 2010, <i>In vivo</i> selection of tumor-targeting RNA Motifs)	Colorectal cancer	p68 RNA helicase	13.8 nM	2'-fluoropyrimidine modified RNA	40	20 minutes
RNA (Mi 2016, <i>In vivo</i> selection against human colorectal cancer xenografts identifies an aptamer that targets RNA helicase protein DHX9)	Colorectal cancer	DHX9 RNA helicase		2'-fluoropyrimidine modified RNA	40	20–30 minutes
RNA (Cheng 2010, <i>In vivo</i> SELEX for Identification of Brain-penetrating aptamers)	CNS delivery	Brain endothelial cells		2'-fluoropyrimidine modified RNA	40	1–3 hours
RNA (Wang 2018, <i>In vivo</i> SELEX of an inhibitory NSCLC-specific RNA aptamer from PEGylated RNA library)	NSCLC	NSCLC cells	9 nM	2'-fluoropyrimidine modified RNA and PEG modification	40	6 hours
DNA (Liu 2018, A novel DNA aptamer for dual targeting of polymorphonuclear myeloid-derived suppressor cells and tumor cells)	Breast cancer	Breast cancer cells + myeloid derived suppressor cells	2.47 nM	Thiophosphate backbone substitution	30	4 hours